

SERUM ALBUMIN MEASUREMENT IN SOME SPECIES
OF DOMESTIC ANIMALS - A DYE-BINDING AND
ELECTROPHORETIC TECHNIQUE COMPARED.

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S U M M A R Y.

This work is centred on the measurement of serum proteins and in particular, albumin, in three species of domestic animals, bovine, ovine and equine. The main methods of protein estimation are reviewed on a historical basis and the various modifications and improvements to each are discussed. During the course of the discussion emphasis is given to the development of methods of protein fractionation by electrophoresis, by the binding of various indicator dyes to albumin and to the results obtained with animal sera.

Although investigated recently in the medical field, very little attention has been paid in the veterinary field to the observation that bromocresol green (B.C.G.) dye is not bound entirely specifically by albumin and that it also undergoes an additional, time dependent reaction with certain other serum globulins. Comparisons between human serum albumin results by this dye-binding method and more specific techniques have shown that albumin levels by the 'immediate' (<30 secs.) reaction with B.C.G. dye are the most accurate. Only one group of veterinary research workers have employed this reaction time when investigating albumin levels in animal serum. The degrees of difference between albumin levels obtained using the 'immediate' and the originally described 10 minute B.C.G. reactions however, have not been demonstrated and part of this work is devoted to the identification of such differences. The effect of using non-specific albumin standards and the reaction of specific purified globulin or albumin-free serum with B.C.G. dye is also investigated.

The remainder of this work is devoted to various aspects of the electrophoretic behaviour of serum proteins and the recognition of the fractions which are clearly identifiable after agarose gel electrophoresis.

The main findings of this work were:-

- (a) There was a positive difference in albumin levels between those obtained by the 'immediate' and by the 10 minute B.C.G. reactions in the three species. The widest of these differences was observed when bovine serum was analysed.
- (b) Only albumin values obtained by the 'immediate' B.C.G. reaction were in good agreement with a more specific method of albumin measurement.
- (c) Electrophoretic albumin values were also in good agreement with those by the 'immediate' B.C.G. reaction.
- (d) Equivalent amounts of purified albumin of each species did not react identically with the dye and the use of albumin standards in the B.C.G. method which were not species specific led to statistically significant discrepancies in albumin results.
- (e) Purified gamma globulin of each species did not react with B.C.G. dye, but other globulins did, although unlike albumin, not on a weight for weight basis. The reactivities of equivalent amounts of alpha and beta globulins of each species appeared not to be identical.

- (f) A set of normal serum protein values was determined for each species using the main methods described in this study.

I N T R O D U C T I O N

Since ancient times the examination of body fluids has been carried out in various forms. Urine and blood, being perhaps the easiest to obtain, have been used extensively in the detection of various diseases and the study of the composition of blood is one which has been of keen interest to both early and modern day clinicians. Each in their own way has relied on their findings to provide or support a diagnosis.

The fluid portion of the blood was originally considered to be composed of a single protein, albumin, which was the early term for proteins and derived from the Latin albus meaning white or in this case, egg white. The earliest form of blood protein fractionation dates back to the last century and was achieved by 'salting out' with ammonium sulphate. The fraction which was precipitated by 50% saturation was termed globulin and that which was not precipitated by 50% but by complete saturation was termed albumin. It was also found that albumin was soluble in water in the absence of salts whereas globulin required the presence of neutral salts for solubility. It was recommended by The Physiological and Biochemical Committees (1908) that the terms albumin and globulin be officially adopted.

Since the very early methods, many other types of protein fractionation have been applied to serum and plasma, the latter terms being those designated for the fluid portion obtained from clotted and unclotted blood respectively. The main difference between serum and plasma is the fact that another protein, fibrinogen, which is utilised in the blood clotting process, is absent in the former. Salts other than ammonium sulphate have been commonly used as protein

precipitants and sodium sulphate in particular was popularly used in fractionation schemes during the first quarter of this century. Howe (1921) stated his preferences for sodium sulphate as a precipitating agent over the ammonium salt and he introduced a fractionation scheme for the estimation of three globulins. Campbell and Hanna (1937) later recommended the use of sodium sulphite and this was also incorporated in the Span-ether scheme of Wolfson, Cohn, Calvary and Ichiba (1948). Of the other salts which have been advocated for use, magnesium sulphate and sodium phosphate were perhaps the most common, although during the past decade 'salting out' methods in general have almost disappeared from routine laboratory use mainly due to their poor resolving power and laborious schedules. Fractionation techniques with organic solvents however, such as the ethanol fractionation scheme of Cohn and associates (1950) in spite of being impractical for general use in the laboratory, still have a commercial application as they are adaptable to large batch processing.

Using one of the earliest forms of electrophoresis, the work done by Picton and Lindler (1892) on the separation of complex compounds under an electrical field eventually led to the development by Tiselius (1937) of a system suitable for use with biological fluids such as serum. This new concept of electrophoresis gave rise to a new terminology with respect to proteins and the serum component with the greatest mobility was identified as albumin. The other three components which were originally observed were termed alpha-globulin, beta-globulin and gamma-globulin in order of decreasing mobility.

As other forms of electrophoresis were developed using various combinations of new support media and buffers, so the number of

proteins detectable in serum and plasma increased. Kunkel & Tiseluis (1952) introduced a simplified procedure using filter paper as a support and at this stage it seemed likely that with some improvements, electrophoresis could be a useful diagnostic tool. Bronte (1952) was one of the first to acknowledge this in his study of liver and bile duct diseases. Using a modified method of Grassman and Hannig (1952), the work of Sunderman and Sunderman (1957) included a statistical analysis of serum protein fractions obtained from approximately 1000 patients and a summary of clinical applications based on the figures obtained from albumin, alpha-1 globulin, alpha-2 globulin, beta and gamma globulins. From this information and from other work, Sunderman (1964) was able to classify 33 known disease categories with their associated electrophoretic findings.

The recognition in the early 1950's that electrophoresis could be a useful diagnostic tool, initiated research into ways of further improving techniques and in particular into other forms of support media with better resolving power. Kohn (1957) described a method for zone electrophoresis using cellulose acetate, a new support medium, which it was claimed was superior to paper in several respects. With some modifications, Kohn (1958) employed this method for micro electrophoresis and immunoelectrophoresis, with some success. In fact a considerable proportion of the electrophoretic work carried out on serum proteins both in the human and veterinary fields in the last 15 years has utilised this support medium.

Of the electrophoretic techniques with much higher resolving powers, the first description was given by Smithies (1955) of zone electrophoresis using starch gel. It was claimed that this technique had a greater ability to separate complex mixtures of proteins

than any other, with albumin and over 30 other protein components being demonstrated in serum. Smithies believed that molecular size was a major factor in determining the type of separation obtained; an observation which was later to be confirmed by other workers.

Ornstein (1964) introduced a new method of disc electrophoresis incorporating an acrylamide gel which gave high resolution with only a brief run of 20 minutes and together with albumin, over 20 protein components of serum were demonstrated on as little as 1 μ l of specimen. In the same year Davis (1964) described a technique applied to human serum using the same type of gel with up to 30 protein components detectable.

A simple technique for agarose gel electrophoresis was described by Johansson (1972) which allowed simultaneous separation of 15 serum specimens. A broad albumin zone with up to 10 other narrower zones were demonstrated in human serum when stained with Amido Black.

Other methods of fractionating serum or plasma proteins which have been developed comparatively recently, but which are still based on the original concept of electrophoresis, include those of isoelectric focusing, gradient gel electrophoresis, sodium dodecylsulphate (S.D.S.) electrophoresis and immunoelectrophoresis. These methods have a research as well as a diagnostic application where proteins of either closely similar molecular size or of very low concentrations are of interest.

The majority of electrophoretic techniques discussed involve some sort of staining reaction and this consists of a coloured dye which is used to make the protein bands visible prior to inspection or to quantitation e.g. by densitometric scan.

On the occasions when the albumin relationship to total globulin is of interest, the former may be measured by another technique in which a dye is involved. The binding of various indicator dyes to albumin has created interest recently and of these, Bromocresol Green has proved to be the most satisfactory for general use in analytical methods.

The considerable attention paid to protein estimations by research workers is perhaps a reflection of the importance of proteins in physiological studies and in disease diagnosis. Fundamental studies have shown that proteins are essential components of all living cells. Among their many complex functions some act as biological catalysts, since all enzymes contain proteins. Some are engaged in transport activities and others fulfil a structural function. Their physicochemical properties give proteins another important role as buffers in the maintenance of acid base balance in the body. Albumin in particular is the most important single major blood protein and among its functions it provides 80% of the colloid osmotic pressure of blood. It also assists in the transport of fatty acids in fat metabolism, acts as a scavenger of heavy metal ions and as a binding or complexing agent for many other molecules and ions.

Their diverse functions thus make determination of various protein levels valuable in the assessment of a variety of conditions and disease processes. The following examples illustrate some fairly common pathological conditions in which protein fractions are altered.

Albumin decrease.

- (a) Inflammatory reaction.
- (b) Malnutrition.
- (c) Water retention.

Alpha-2 globulin increase.

- (a) Acute inflammatory response.

Beta-1 globulin increase.

- (a) Iron deficiency.

Beta-2 globulin increase.

- (a) Hypercholesterolaemia.
- (b) Chronic inflammatory response.
- (c) Biliary obstruction.

Gamma-globulin increase.

- (a) Monoclonal gammopathies. e.g. multiple myeloma.
- (b) Polyclonal gammopathies. e.g. chronic infections.

(Laurell, Jeppson and Tejler (1978)).

In the veterinary field the use of total protein, albumin measurement and protein electrophoresis as a diagnostic and research tool has won wide recognition in the last 20 years, although considerable work had been carried out prior to this by Deutsch and Goodloe (1945), Ashton(1957a&b)and Hickman and Smithies (1957) among the major contributors.

There is no doubt that protein studies have an important role to play today in diagnosis. Investigation and refinement of the techniques involved is consequently of considerable importance.

CHAPTER I

REVIEW OF THE LITERATURE

METHODS OF TOTAL PROTEIN DETERMINATION IN SERUM AND PLASMA.KJELDAHL DETERMINATION OF PROTEIN N.

The widely accepted reference method for the determination of total protein in biological material is based on the Kjeldahl (1880) procedure. This procedure, which determines the protein N content of the material is usually only performed in the laboratory to standardise or evaluate other methods owing to the complexity of the technique and the length of time required for completion (Henry, 1964).

The principle of the digestion technique originally described by Kjeldahl is based on the fact that N-containing compounds can be converted to NH_4^+ and this, when treated with alkali produces NH_3 . Kjeldahl achieved this by oxidation in an acid digestion mixture with potassium permanganate included to assist in the oxidation of alkaloid compounds. The NH_3 produced is absorbed in a known volume of standard acid and the excess determined by titration.

Since the original principle was established, most of the modifications to the method have taken the form of additions or substitutions to the digestion mixture, although they invariably include sulphuric acid, as did the original. Some modifications include the addition of another oxidising agent to complete the digestion and clear the mixture. To raise the boiling point of the mixture, potassium sulphate has been proposed and as oxidising agents, potassium persulphate and hydrogen peroxide. Kjeldahl included phosphoric acid in his digestion mixture but other alternative agents have been successfully employed such as the metallic catalysts selenium, mercury and copper. (King, Haslewood and Delory, 1937; Campbell and Hanna, 1937; Wagner, 1940 and Hiller, Plazin and Van Slyke, 1948.)

After the conversion of NH_4^+ to NH_3 some of the modifications to the original procedure advise steam distillation of the NH_3 into a flask containing boric acid. This can then be titrated with standard HCl as in the method of Wagner (1940). The advantages of NH_3 distillation into boric acid instead of into a measured amount of standard acid are discussed by Wagner and are outlined by Cannon, Olitzky and Inkpen, (1974). Alternatively, the NH_4^+ formed by the digestion may be determined colorimetrically with the use of Nessler's reagent (Koch and McMeekin, 1927; Harrison, 1957), or by the Berthelot reaction (Berthelot, 1859; Fawcett and Scott, 1960; Chaney and Marbach, 1962 and Leffler, 1964). Henry (1964) considered the latter determinations to be less satisfactory than the titrimetric analysis owing to their poorer accuracy and precision.

In Kjeldahl procedures, in order to determine the actual protein content of the material under analysis it is first necessary to ensure that a complete or at least a reproducible recovery of the protein N is achieved. In the case of serum or plasma this can be managed quite satisfactorily. If biological material such as serum or plasma is under analysis, it is assumed that the N content of the various proteins in the material is taken to be constant since total N is converted to total protein with the aid of a factor. Although the protein factor in general use for serum proteins had an obscure origin, it has nevertheless been in favour for a considerable period. It assumes that proteins derived from biological sources had an N content of 16% by weight and that the figure was multiplied by 6.25 to obtain the total protein content of the material. A factor of 6.54 was later recommended by Chiaraviglio, Wolf and Prentiss (1963) and Sunderman (1964a). These workers found the N content of

total proteins in serum to be 15.3%, a figure which was stated to be significantly lower than previously reported.

Higher figures are quoted for protein conversion factors from N content, but they relate to protein types with higher lipid and carbohydrate proportions, which contain less N. The usual practice however, is to disregard the non-protein moieties which may be present and to base the calculation only on the protein moieties. (Strickland and associates, 1958, Sunderman, 1964 and Peters, 1968).

PROTEIN DETERMINATION BY THE BIURET REACTION.

Owing to its simplicity and favourable reproducibility, the biuret method for the determination of serum and plasma proteins has been the most commonly used of the many colorimetric methods proposed for use in the clinical laboratory.

When urea is heated to 180°C it decomposes to produce a substance called 'biuret' ($\text{NH}_2\text{.CO.NH.CONH}_2$). When this is treated with copper salts in alkaline solution, a violet coloured complex is formed. This colour reaction is given by substances containing two $-\text{CONH}_2$, $-\text{CH}_2\text{NH}_2$, $-\text{C}(\text{NH})(\text{NH}_2)$ or $-\text{CSNH}_2$ groups directly linked or through a C or N. The reaction also occurs with peptide structures containing at least two peptide linkages. The reaction with the peptide bonds of proteins provides the basis for the determination of proteins in serum and other biological fluids (Peters, 1968).

Rose (1833) first mentioned the biuret reaction and later Wiedemann (1848) studied the reaction of copper with biuret. The first application of the biuret reaction to proteins was made by Ritthausen & Pott (1873). Of the early techniques which were applied to serum, most used a considerable excess of copper salt and under the

alkaline conditions employed, about 3% of the copper precipitated as $\text{Cu}(\text{OH})_2$ which had to be removed by filtration or centrifugation. Kingsley (1942) devised a simplified biuret procedure in which a single reagent was required instead of the combination reagent previously used in other methods. Kingsley used a low concentration of copper salt which was kept stabilised in strong alkaline solution and this avoided precipitation of $\text{Cu}(\text{OH})_2$. The main disadvantage of this type of reagent was its limited stability period, since cupric salts are susceptible to autoreduction.

Several later modifications were proposed to stabilize the biuret reagent and permit the use of lower alkalinity without the formation of $\text{Cu}(\text{OH})_2$ precipitates. Weichselbaum (1946) proposed a biuret reagent in which sodium potassium tartrate and potassium iodide were used to act as a stabilizer and anti-autoreduction agents respectively. This reagent was later criticised as being unstable after long storage (Henry, 1964). Gornall, Bardawill and David (1949) also produced a tartrate stabilized biuret reagent with a tartrate to copper sulphate ratio of 3 : 1 which proved more satisfactory. He found that the inclusion of potassium iodide was unnecessary if high grade copper sulphate was used in the preparation of the reagent. (Also De la Hueraga, Smetters & Sherrick (1964))

It was later noted (Henry, Sobel and Berkman (1957), Hussain, Shah and Chaudhuri (1961)) that Benedict's qualitative glucose reagent proved to be a highly satisfactory biuret reagent and techniques employing this reagent are still widely used in laboratories. The reagent has a low alkalinity having Na_2CO_3 instead of NaOH , contains citrate as a stabilizer and is stated to be stable indefinitely at room temperature. NaOH is introduced at a separate

stage as a 3% solution. The absorption maxima of the protein biuret complex are 545 nm against a biuret blank and 580 nm against a water blank. A fourfold increase in sensitivity can be achieved by measurement at 300 nm but any slight turbidity interferes markedly in this region. The colour produced obeys Beer's Law and is stable for several hours, reaching its maximum after 15 minutes. In the absence of interference from turbidity and haemolysis, agreement between this biuret technique and that of the Kjeldahl reference method is generally favourable (Henry et al, 1957). If necessary, interferences due to turbidity can be removed by ether extraction. If the haemoglobin concentration is known, a correction can be applied to compensate for any haemolysis present.

OTHER METHODS FOR THE DETERMINATION OF TOTAL PROTEIN IN SERUM OR PLASMA.

S.G. measurements.

A method which was designed mainly for field use was introduced by Van Slyke and associates (1950). Total protein is computed by suspending drops of serum or plasma into a graded series of cupric sulphate solutions each of known specific gravity. A rise or fall of each discrete drop of copper proteinate indicates the S.G. relative to the solution. The method was later criticised by other workers (Bernstein, 1954; Henry, 1964) who stated it to be unreliable with abnormal protein levels, lipaemic sera and those with high levels of glucose or urea.

A major factor in determining the accuracy of the method is the number of standards employed and it is usual for 21 to be included, covering a range of S.G. 1.015 - 1.035, corresponding to total protein concentrations of 30 - 108 g/l.

Refractive index.

The measurement of refractive index as a means of determining the protein content of a solution was introduced by Reiss (1902). Robertson (1915); Alder (1928) and Loewe (1933) used a similar technique with some modifications. These early workers all used the Zeiss-Pulfrich immersion refractometer which had a scale calibrated in grams of protein per 100 ml. Protein values quoted using the original Reiss refractometer were higher than those obtained using the standard Kjeldahl method (Alder, 1928) and at the time this was considered to be the main criticism of the technique.

Sunderman (1944) suggested a statistical regression formula to be used in conjunction with refractometry and the results obtained gave close agreement with total protein using Kjeldahl and biuret methods. More recently a new regression formula for refractometry of total protein in serum was proposed by Drickman and McKeon (1962).

The main advantage of refractive index measurements is primarily that they are direct, rapid and usually very effective with clear serum. Discrepancies can occur when substances such as bilirubin, cholesterol, triglycerides and glucose are present in large quantities as the determination of protein by this method is dependent upon the non-protein content of the material being constant. (Henry, 1964 & Naumann 1964.)

Phenol method.

Folin and Denis (1912) found that a phosphotungstic-phosphomolybdic acid reagent, when reacted with substances containing a phenolic group, produced a blue colour. This was the basis of the colorimetric method introduced by Wu (1922) for the determination of plasma proteins, which was later adapted for use with urine and

spinal fluid. Folin and Ciocalteu (1927) produced a reagent which lessened earlier problems with turbidity. Previously the occurrence of turbidity was attributed to the formation of insoluble sodium salts. The reagent of Folin and Ciocalteu contains more soluble lithium salts instead of sodium salts. Some turbidity, however, may develop even with this reagent.

The phenol method has never really been widely recommended for routine use with serum or plasma for several reasons. The phenol reagent is not specific for protein and errors can arise if the patient is toxic or has high blood levels of other phenolic compounds. Tyrosine, tryptophane and cysteine of proteins are responsible for the colour produced when the phenol reagent is reacted with serum or plasma. The usual reference standard employed is a solution of tyrosine and the calculation is based on the chromogenic equivalent in terms of tyrosine. It has been pointed out, however, that the tyrosine equivalents for albumin and globulins are not identical and it is also possible for the tyrosine equivalent of proteins to be altered in certain pathological conditions (Greenberg, 1929; Tuchman and Sobotka, 1932).

Substances which react with cupric ions in alkaline solution to give a positive biuret reaction, react in a similar manner with Folin and Ciocalteu reagent in the presence of cupric ions (Herriot, 1941). By including cupric ions in the phenol reaction, its sensitivity can be increased to almost one hundred times that of the biuret reaction, making it suited for use with more dilute fluids such as C.S.F. and solutions of protein extracts where the total protein concentration is very low.

Ultraviolet absorbance measurements.

The majority of proteins present in body fluids are colourless but absorb light in the U.V. range of 200 - 280 nm. Absorbance in the range 260 - 280 nm. is due to the presence of aromatic amino acids, tyrosine, tryptophan and phenylalanine. The magnitude of absorption in this region is dependent upon the proportion of these amino acids which may be present in the material. Strickland et al, 1960; Peters, 1968, noted the wide variations in tyrosine and tryptophan content between albumin and the other protein fractions.

Although there can be wide variations in specific absorptivity of various proteins, this can be reduced when measurements are made between 200 and 250 nm. Absorption in this region is attributed mainly to the peptide bonds of proteins.

Owing to the high relative ratio of proteins to possible interfering substances in serum, U.V. absorbance measurements of diluted serum provide a satisfactory means of determining the protein concentration. Waddell (1956) proposed that the difference in absorbance of diluted serum at 215 and 225 nm, should be used to determine the protein concentration instead of measurement at a single wavelength. Protein determination by absorbance difference greatly reduces the error from non-protein serum constituents. Waddell reported a favourable comparison of results achieved using his own versus Kjeldahl and biuret techniques. Murphy and Kies (1960) achieved excellent results using the Waddell method in their determinations of protein in dilute solutions.

The usual 1,000 or 2,000 fold dilution as applied to serum can not be employed when analysing other body fluids with much lower

concentration, such as C.S.F. It is therefore not possible to minimise the effects of any interfering substances which may be present. (Patrick and Thiers, 1963.)

Dye-binding.

The dye-binding effect of proteins was first noted by Sorenson (1909) and termed the 'protein error' of indicators. Because various proteins bind differently to form complexes which cause a change in colour of a pH indicator, this method is not recommended for determination of total protein in serum or plasma. The application is however suited to the quantitation of individual proteins such as serum albumin and this approach will be considered more fully in a later section.

CHAPTER II

REVIEW OF THE LITERATURE

F R A C T I O N A T I O N O F P R O T E I N S

As indicated in the introduction, biological fluids such as serum and plasma contain a heterogeneous mixture of many proteins. Chemical or physical separation of these is termed 'fractionation'.

CHEMICAL METHODS

The general principles of salt fractionation methods have already been outlined as have the reasons for their gradual phasing out from regular laboratory use.

Over the years, comparisons have been drawn between salt fractionation and electrophoretic techniques. Taylor and Keys (1943) stated that the so-called albumin fraction obtained after serum precipitation with 22.5% sodium sulphate after the method of Howe (1921), was invariably greater than the albumin level as determined by electrophoresis. Dole and Braun (1944), Peterman, Young and Hogness (1947) and Cohn and Wolfson (1947) arrived at essentially the same conclusion in analysing the serum levels obtained from normal individuals.

Pillemer and Hutcheson (1945) used aqueous methanol as a precipitating reagent at pH 6.7 - 6.9, ionic strength, 0.03 and at a temperature of 0°C. Good agreement for albumin values was obtained using this technique versus electrophoresis. The work of Martin and Morris (1949) confirmed these results.

Other favourable comparisons between salt fractionation and electrophoretic methods for the measurement of albumin and globulin in human serum were completed by Majoor (1947), Milne (1947), Wolfson et al (1948) and Levin, Oberhalzer and Whitehead (1950).

Perk and Lobl (1960) studied the albumin levels obtained from

ewes, rams and lambs of various age groups, using both chemical and electrophoretic techniques. They compared their results obtained by paper electrophoresis with those by the chemical method of Kingsley (1939) as modified by Weichselbaum (1946). Perk and Lobl observed that the albumin values obtained by chemical determination were consistently higher than those by electrophoresis. The figures given for lactating ewes (3 years old) were total protein (mean values) of 73.6 g/l, albumin by chemical method 32.6 g/l and by paper electrophoresis 30.7 g/l.

Since 1950 few improvements have been made to the earlier salt fractionation methods with the exception of the Reinhold (1953) modification, which was later recommended for routine use (Varley 1963).

The advent of electrophoresis however afforded a simple replacement for the various salt fractionation methods, as a means of determining the albumin/globulin ratio as well as other information on alpha, beta and gamma globulins. Electrophoresis has now become well established in clinical laboratories as the method of choice for protein fractionation and is normally used in conjunction with a simple biuret method for the determination of total protein.

ELECTROPHORESIS

Michaelis (1909) is on record as being the first to use the term electrophoresis, to describe the migration of colloids under the influence of an electrical field. (Sargent and George, 1975). The term ionophoresis has been used (Martin and Synge (1945)) to describe the movement of certain ions under similar conditions, but is not a term that is regularly used today. A more modern description of the term electrophoresis would be that it refers to the separation

of components in a mixture, based on the principle that a charged molecule or particle will migrate either towards the anode or cathode when placed in an electrical field. The rate of migration is directly related to the size of its net charge.

Many new forms of preparative and analytical electrophoresis have been developed in the last decade but the two main recognised types, based on the original concept, are those of boundary and zone electrophoresis.

MOVING BOUNDARY ELECTROPHORESIS

As outlined earlier, the first moving boundary method was introduced by Picton and Lindler (1892) and was later improved by Tiselius (1957). In this method proteins are optically measured as they migrate through a buffer solution. Essentially, changes in refractive index are recorded with the aid of very sensitive Schlieren optics which allow the zones of separation to be scanned, thus providing a means of measuring the rate of change in concentration.

This is usually performed in a 'U'-shaped tube where the protein solution is layered under a buffer solution. Density differences ensure that the system is stabilised and migration can take place in free solution (Tiselius, 1957). The theory of migration boundaries in electrolyte solutions is complex and is detailed by Longworth and MacInnes (1940) and Dole (1945).

Deutsch and Goodloe (1945) in their electrophoretic survey of plasma from various species of animals conducted their experiments in a modified Tiselius apparatus and using Schlieren optics the moving boundaries were photographed and enlarged. After projection and tracing the areas under the curves were measured planimetrically

and the percentage composition of each fraction was calculated from these figures. The results obtained were compared to those from human plasma.

Continuous developments in the field of zone electrophoresis over the past twenty years have given this procedure many advantages over moving boundary methods as a means of protein separation. Moving boundary methods are now generally regarded as being only of historical interest and more detailed consideration will be given to zone electrophoresis and support media.

ZONE ELECTROPHORESIS

In zone electrophoresis, the zones have a relatively high density compared to the medium and Tiselius (1957) outlined the reasons for using stabilising materials such as paper, starch powder or cellulose. In recent years agarose and polyacrylamide have also been employed in this respect (Ornstein, 1964, Johansson 1972).

It has been previously stated that electrophoresis is based on the fact that a charged particle will migrate between two electrical poles at a rate that is directly related to its net charge. On this basis it is thus possible to separate substances of close similarity which may not be separated by other means.

Some general requirements are necessary and indeed are applicable to all forms of zone electrophoresis. It is proposed to outline these before discussing the various types of support media and results obtained.

Effect of pH

Particles to be separated by electrophoresis must first be capable of carrying an electrical charge. Proteins are suitable

in this respect since they consist of charged molecules made up of dipolar amino acids with both acidic COO^- and basic NH_3^+ groups (Tiselius, 1957). Their net charge is dependent on pH and the point at which this charge is reduced to zero is termed the 'isoelectric point'. (Where there are equal numbers of COO^- and NH_3^+ groups). Ideally there will be no migration of a protein in an electrical field if the pH of the buffer is at the isoelectric point of the protein. (This is also the region of least solubility of a protein).

If the buffer has a pH that is alkaline to its isoelectric point, the protein will have a net negative charge, owing to COO^- groups being predominant and a net positive charge if the converse is the case. As a rule most separations for clinical purposes are performed with alkaline buffers since most proteins tend to absorb the support medium in acid buffers. (Meltzer, 1968).

Buffer

In general the electrophoretic buffer should have good conducting qualities and be inert in the electrophoretic process although certain buffers can complex some media thus influencing the separation (Smith, 1976). The buffer is normally used at a concentration of 0.05 - 0.10 M and the conductivity is determined by its ionic strength.

As a generalisation it may be stated that the use of buffers of high molarity result in sharper zones of separation than buffers of low molarity, although the separation time is longer in the former case (Sargent & George 1975).

The ionic strength may be calculated using the following equation:-

$$\mu = \frac{1}{2} \sum mc^2$$

where μ is defined as ionic strength

m is defined as molarity (gram ions/litre)

or molarity (gram ions/litre solvent)

and c as the charge on the ion.

(Smith, 1976).

Faster particle migration rates can be achieved with the use of buffers of relatively low ionic strength which offer a higher resistance to current flow. In electrolytic solutions, charged particles attract a cloud of positively charged ions which tends to lower the net charge on the molecule. As the ionic strength is lowered, the cloud becomes more diffuse which results in a greater net charge and faster migration rate.

However this also allows more diffusion in the medium, giving poorer resolution, but compensation for this can be achieved by increasing the voltage. Since the voltage is increased, migration rates are speeded up and this effectively allows less time for the particles to diffuse (Millipore, 1970).

Voltage current and heating

Having established that the particles to be separated are capable of carrying an electrical charge, an electrical field is required to induce the charged particles to migrate. The electrical field is a function of the voltage applied to the ends of the electrophoretic track and is expressed in volts per centimetre (V/cm). (Millipore, 1970). Since the voltage is the driving

force, it should be selected high enough to give fairly rapid separation, although too high a voltage can result in excessive heat production with continuous consequent distillation off the electrophoresis strip (Smith, 1976). Based on Ohm's Law, $\text{Voltage} = \text{Current} \times \text{Resistance}$; since initially during separation the resistance is relatively constant, a higher voltage results in greater current, thus more heat is produced ($\text{Voltage} \times \text{Current} = \text{Watts}$).

The prevention of excess heat can be achieved either by cooling the system or by reducing the ionic strength of the buffer. Since the current is conducted by ions in solution, if the concentration of ions is decreased then the resistance of the system is effectively increased (Millipore, 1970).

Electroendosmosis

In electrophoresis, when differing chemical substances such as water and certain types of support media come into contact they may become relatively charged with the water becoming the positive. Although the support medium is fixed, the water is free to move and so it streams towards the cathode, carrying the buffer salts and components of the mixture with it. In protein electrophoresis this stream is in the opposite direction to that of the migration and thus slow moving gamma globulins are carried back beyond the origin. (Smith, 1976).

Some commonly used types of support media such as agarose gel and polyacrylamide gel have low endosmotic rates in comparison to paper and agar (Corkhill, 1977).

Support media

The purpose of zone electrophoresis is to permanently separate the specimen into discrete bands on a support medium with a view to further analysis either soon after separation or at a later date and if possible, to provide a permanent record of the investigation.

Both in the medical and veterinary fields several forms of support media are currently in use for the examination of clinical material and some of the more commonly used types will be outlined here. The earlier types such as paper and starch gel have now largely been replaced by more convenient forms which allow rapid, well defined separations and have favourable handling characteristics.

Paper: In the veterinary field, early work on paper electrophoresis was carried out by Boguth (1954) and Kao, Reagan and Brechner (1954). The paper was intended to act as an inert support for the buffer and electrophoresis was usually performed in a horizontal type chamber. Perk and Lobl (1960) used such a system in their analysis of normal sheep serum proteins, with Whatman No. 1 filter paper acting as support. Although perhaps the simplest of electrophoretic methods and the easiest media to handle, the use of paper for protein separation is not now generally recommended. This is mainly due to the fact that paper is not a particularly homogeneous medium in terms of pore (capillary) sizes and also tends to absorb materials especially proteins (Sargent and George 1975).

Starch gel: Smithies (1955) introduced a method of zone electrophoresis using starch gel as the support media. This

material is prepared by heating a uniform suspension of hydrolysed starch in an appropriate buffer and is not just an inert support such as paper of cellulose acetate. The material exerts a direct effect on the separation process and components of a mixture such as serum or plasma migrate through the pore structure of the gel which acts as a molecular sieve, such that those proteins of a similar charge to mass ratio separate if their molecular size is different.

Ashton (1957a) investigated the resolution of serum proteins in cattle, pigs, horses and dogs using the method of Smithies and also Ashton (1957b) studied the serum protein differences in several breeds of cattle.

Cellulose acetate: A convenient replacement for paper as a support medium for the study of proteins in the medical field, introduced by Kohn (1957) was cellulose acetate membrane (C.A.M.) This was described as a medium of fine, microporous structure which offered several advantages over the earlier paper types. The use of cellulose acetate was claimed to overcome the previously troublesome 'tailing' which was common in paper electrophoresis. Adsorption was stated to be minimal with this new medium and a sharp separation of well defined bands was thus possible since the background was colourless. Background clarity greatly improves the accuracy of any subsequent quantitative determination and coupled with other advantages of rapid separation, staining, drying and washing, the complete electrophoretic procedure could be completed in one hour. In addition, it was discovered that after processing cellulose acetate strips, it was possible to render the

background transparent by immersion in a suitable clearing agent. This is particularly useful if densitometric scanning is required or if strips are to be photographed.

Matthews, Kaneko, Loy, Cornelius and Wheat (1966) were amongst the first to employ this new medium in the veterinary field, in their study of compartmentalisation and turnover of ^{131}I - labelled albumin and gamma globulin in horses.

Polyacrylamide gel: Raymond and Weintraub (1959) were the first to suggest polyacrylamide gel as a medium for electrophoresis. At that time starch was the major support medium in use but as it was later pointed out, results obtained with gels prepared from both starch and agar were highly dependent upon the quality of each batch (Sargent and George, 1975). Unlike natural products, polyacrylamide is an entirely synthetic polymer and can be prepared reproducibly from simple chemicals of high purity. Polyacrylamide has no charged groups present and so electroendosmotic effects are minimised. Gels of different pore sizes can be constructed producing a sieving effect by which groups of molecules can be separated according to size. Electrophoretic resolution with this medium is superior to that of others previously mentioned and its properties were recognised by Ornstein (1964) and Davis (1964) who developed a technique of polyacrylamide disc gel electrophoresis. The authors were able to demonstrate between 20 and 30 protein components separated from only 1 μl of serum in a system containing polyacrylamide gels of different pore sizes. Hyslop (1972) applied an improved system of electrophoresis on polyacrylamide gel to studies on the sera of different species.

Agarose gel: A technique of agarose gel electrophoresis was described by Johansson (1972) in which simultaneous separation of up to twenty protein specimens was carried out in agarose gel on water cooled plates; the procedure taking less than one hour.

Agarose is the almost neutral product obtained when seaweed agar is fractionated. One fraction has a high content of sulphate and hydroxyl groups and is called agarpectin, while the other, agarose, has a very low proportion of these groups present (Jeppsson, Laurell and Franzén 1979).

Since commercially available preparations of agarose still contain traces of sulphur up to 0.2%, this gives rise to a slight endosmosis on electrophoresis, but this is quite satisfactory for use in the separation of proteins from serum and plasma. In fact, a certain degree of electroendosmosis may be advantageous when the gamma globulin region is to be inspected (Johansson, 1972). In its earliest introduction into the veterinary field Carlstrom and Liberg (1975) slightly modified the method of Johansson (1972) and utilised it in their study of cattle serum proteins.

OTHER FRACTIONATION TECHNIQUES

Affinity chromatography

This is a type of adsorption chromatography in which sample components are isolated and separated according to their different biological specificity. The molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support matrix.

The first application of affinity chromatography dates back to the early 1900's when amylase was selectively adsorbed on to

insoluble starch. It is only comparatively recently however that the technique has become established in laboratories. This was mainly due to the complex organic chemistry involved in the synthesis of a reliable matrix and the attachment of ligands co-valently. The widespread adoption of affinity chromatography in recent years commencing with the work of Axen, Porath and Ernback (1967) is a reflection of its success in achieving rapid separations, which were previously difficult or more time consuming to obtain with conventional techniques. Highly selective, affinity chromatography can be used for purifying substances from complex biological mixtures, separating native from denatured forms of the same substance and recovering small amounts of biological material from large amounts of contaminating substances.

As indicated, the work of Axen et al. (1967) heralded the beginning of affinity chromatography as a routine separation technique. They concluded that molecules containing primary amino groups could be coupled to polysaccharide matrixes. One example of a highly effective chromatography matrix is that of Cibacron Blue F3G-A dye (Ciba-Geigy) coupled to crosslinked agarose beads. The conjugate has a biospecific affinity for nucleotide requiring enzymes probably due to the structural similarity between its blue chromophore and nucleotide factors, Wilson (1976).

The conjugate also has the ability to selectively absorb albumin from serum and in fact, the binding is so strong that a high concentration of salt is required for desorption.

The blue dye has also been coupled with polyacrylamide (Kopperschlager et al 1971); dextran gels (Bohme and associates, 1972)

dextran/agarose (Witt and Roskoski, 1975), CNBr - activated agarose, (Ryan and Vestlung, 1974) and agarose, (Heyns and de Moor, 1974) for example. Each of these dye conjugates have been used in the purification of various enzymes. The Blue Dextran/Agarose conjugate was favoured by Travis and Pannell (1973 and 1974) in their selective removal of albumin from plasma and also by Wille (1976). Travis, Bowen, Tewksbury and Pannell (1976) later described an improvement to their earlier affinity gel which resulted in a greater binding capacity for plasma albumin.

'Rocket' Immuno-electrophoresis

Alternative terminologies for this immunological method are electroimmunoassay and antigen electrophoresis in antibody-containing agarose gel.

The basic principle used by Heidelberger and Kendall (1932) for the estimation of serum proteins by quantitative precipitation requires specific antisera to each individual protein of interest. The method represents a simple, quick and reproducible method for determination of a single protein in a protein mixture, Weeke (1973). The predecessor to Rocket techniques was the method of Mancini et al. (1964) which was based on radial diffusion of antigen in agar containing specific antibodies. Although technically simple and considered exact enough for clinical work, each determination could take several days to complete. Apart from the advantage of speed Laurell (1966) claimed that rocket immuno-electrophoresis had an error of only a few per cent, was suitable for serial analysis and required only 2 to 0.5 μ g protein antigen. The amount of antiserum required was less than for radial immunodiffusion techniques.

Rocket immunoelectrophoresis is normally performed in agarose gel containing monospecific antiserum. Identification of a single protein is given by the rocket shaped precipitate formed and its quantitation can be based upon measurement of the height of the precipitate compared to that of suitable reference standards. Corcoran and Durnan (1977) included the rocket technique in their comparison of human serum albumin values, obtained with a modification of the bromocresol green (B.C.G.) dye binding technique of Doumas, Watson and Biggs (1971). A favourable comparison between these two techniques was achieved if results from the dye-binding technique were calculated after 10 seconds reaction between dye and serum.

Gustafsson (1978) arrived at a similar conclusion when he compared the Laurell (1972) rocket technique for human serum albumin with that of the Doumas et al. (1971) bromocresol green (B.C.G.) dye technique and a B.C.G. technique modified for use with a reaction rate analyser. If the reaction times between serum and dye were prolonged i.e. between 10secs and 15 mins. the results obtained from the B.C.G. method did not compare well with those from the more specific immunological method. Albumin was shown to be responsible for the 'immediate' reaction i.e. less than 10 secs. while other proteins contribute to the reaction over a longer period. Results from a similar type of comparison by Webster (1977) also support these conclusions.

Isotachophoresis

Although recognised at the end of the last century, this variant has not found routine application until comparatively recently. Isotachophoresis is an electrophoretic technique where

sample components achieve separation on the basis of their net mobility, Svensson (1961), Everaerts, Mulder and Verheggen (1974). Both analytical and preparative systems are now available. Column isotachopheresis may be carried out in a polyacrylamide gel of relatively low concentration which aids the stabilisation of the separated zones during the final elution from the column. Owing to its concentrating effect isotachopheresis gives a high resolving power with no diffusional broadening of zone boundaries.

Isoelectric focusing

The theoretical basis of this method was laid down by Svensson (1961) but it was the early work of Kolin (1954) on the creation of natural pH gradients which led to later major advances in the development of synthetic ampholytes (Vesterberg and Svensson 1966). Ampholytes (e.g. 'Ampholine', Pharmalyte' trade names) are a series of zwitterions with different overall pK values giving rise to different isoelectric points (pI).

The principle of isoelectric focusing is based upon the migration of a given protein to a fixed point within a stable pH gradient under the influence of an electrical field. The point at which the migration ceases is the isoelectric point (pI) of the protein and corresponds to the pH at which charges are equal (i.e. neutral). In a gel with a stabilized pH gradient, once a protein has reached its pI it will no longer be influenced by the electrical field and will remain at its fixed position almost indefinitely. Thus isoelectric focusing can be considered as a static technique, the final position of each protein not being dependent upon migration time or point of application but on the net surface charge of each protein. One

major advantage of this method is that a difference in isoelectric point of only 0.01 pH units is sufficient to achieve separation. (Svensson 1961).

The ampholytes referred to, are produced in a polymerisation reaction between acrylic acid and many polyamino compounds as described by Vesterberg (1969).

Gradient gel electrophoresis

Two examples of methods which have been developed comparatively recently and which effectively separate proteins on the basis of size alone, are those of Gradient Gel Electrophoresis and Detergent Gel Electrophoresis.

The most important requirement for this form of electrophoresis is that a suitable pore gradient gel be prepared.

The preparation of the gel is such that an increasing concentration of polyacrylamide decreases the size of the pores in the direction of electrophoretic mobility. When a mixture of protein molecules of different size is subjected to electrophoresis in such a gel, the molecules initially begin moving at a speed determined by charge when the electrical field is applied. As the pore size of the gel decreases however, the larger molecules begin to slow down and are finally immobilised at the point in the gel where the pore size is such as to prevent further passage.

It is claimed that gradient gel electrophoresis is one of the highest resolution methods of protein separation because of its ability to produce sharply stacked bands and separation of serum into 43 bands has been reported. Examples of this method can be found in the work of Slater (1965), Margolis and Kenrick (1968),

Grossbach and Weinstein (1968), Caton and Goldstein (1971) and Petri (1972).

Detergent gel electrophoresis

This technique is often referred to as SDS Electrophoresis after the anionic detergent sodium dodecyl sulphate. Among the first to use this particular form were Summers, Maizel and Darnell, (1965).

Other types of detergents have also been incorporated in gel buffers as a means of liberating proteins from complexes and eliminating aggregates e.g. Jackson and Lawton (1958), used the cationic detergent cetyltrimethylammonium bromide (CTAB) and Schneidermann (1965) used Triton X - 100.

The principle behind the method is that proteins can be separated on the basis of size alone if they are first solubilised with the detergent sodium dodecyl sulphate (SDS). SDS binds to the protein molecules converting them to a rod-like shape, and masking their own charge with its negative charge. Shapiro, Vinuela and Maizel (1967) were the first to recognize this phenomenon.

When subjected to polyacrylamide gel electrophoresis, the charge densities of each protein are now equal and are separated purely on the basis of size by the molecular sieving effect of the gel. In recent years this method has been frequently used for determination of protein molecular weights, e.g. Weber and Osborn (1969). It is claimed that SDS - polyacrylamide systems enable molecular weights to be determined to within 10% for proteins larger than 10,000 molecular weight.

SUMMARY OF METHODS OF PROTEIN SEPARATION

1. By net mobility = Isotachophoresis.
2. By pI = Isoelectric focusing.
3. By size = Gradient electrophoresis and
detergent gel electrophoresis.
4. By charge = Zone electrophoresis.
5. By bio-specific affinity = Rocket I.E.P. affinity
chromatography.

CHAPTER III

REVIEW OF THE LITERATURE

A L B U M I NDYE-BINDING TECHNIQUES

The principle of various dye binding techniques for the determination of serum or plasma albumin depends on the fact that when protein is added to a solution of certain dyes, a protein/dye complex is formed which exhibits different optical properties to those of the free dye (Sobotka and Stewart, 1965). This has been termed the 'protein error' of indicators and some such dyes have been considered reasonably specific for the measurement of albumin.

Grollman (1925) claimed to be the first worker to undertake quantitative analysis to determine the nature and factors influencing the combination of an indicator dye (phenol red) with proteins.

Klotz, (1946) investigated the binding of organic ions by proteins using crystalline bovine albumin and two sulphonate ions, methyl orange and azosulphothiazole. It was suggested that in the interaction of sulphonate ions with albumin, the points of attachment are probably the cationic groups in the molecule. It was observed that the maximum number of bound anions, 22, corresponded roughly to the number of arginine residues in the bovine albumin molecule.

Bracken and Klotz (1953) introduced a method for the direct determination of albumin in which serum was added to a solution of methyl orange buffered at pH 3.5. The resulting decrease in absorbance at 550 nm provided a measure of the albumin concentration in the serum. It was reported that the methyl orange procedure over estimated albumin, especially at low levels and later Rosenberg, Laver and Lyons (1955) found that beta lipoproteins, alpha-1 and alpha-2 globulins were also capable of binding methyl orange, which confirmed the lack of specificity of the method.

Interest in the technical aspects of the interaction of protein and dyes was nevertheless stimulated after the introduction of this method and Rutstein, Ingenito and Reynolds (1954) and Blondheim (1955) studied the dye binding capacity of serum albumin using 2 - (4' hydroxybenzeneazo) benzoic acid (H.A.B.A.) and phenol red respectively.

Wrenn and Feichtmeir (1956) proposed improvements to the method of Rutstein et al, which included stabilization of the dye reagent with formaldehyde. Among further modifications to the H.A.B.A. method, those of Goodwin (1964) were accompanied by a comparison of results obtained using this method, a methyl orange method and paper electrophoresis. Later modifications to the H.A.B.A. method by Ness, Dickerson and Pastewka (1965) included replacement of the original pH 5.0 acetate buffer system with a pH 6.2 phosphate buffer to reduce turbidity and the measurement of absorbance at 485 nm to increase sensitivity. The temperature of the H.A.B.A./albumin reaction was controlled owing to its thermolabile nature.

Pastewka and Ness (1965) assessed the suitability of various serum albumin products as standards for use with the H.A.B.A. quantitative dye procedure. It was concluded that albumin-Fraction V appeared suitable as standard reference and control material. The H.A.B.A. dye procedures, in spite of their greater specificity for albumin over methyl orange, suffer from low sensitivity and have the disadvantage of interference from various materials such as salicylate, sulphonamides, penicillin and conjugated bilirubin (Arvan and Ritz, 1969). Heparin anticoagulant causes turbidity which also interferes with the assay (Arvan and Ritz, 1969).

Rodkey (1965) introduced a method for the direct spectrophotometric determination of albumin in human serum, which was based on the decrease in absorbance of a neutral buffered solution of bromocresol green (B.C.G.) when albumin combined with the indicator. It was claimed that the method was more sensitive than previous spectrophotometric procedures and because of the very high association constant of the albumin - bromocresol green complex, competitive interference from other constituents was not a problem.

Doumas, Watson and Biggs (1971) described a method for the measurement of serum albumin in which the addition of albumin to a solution of B.C.G. in 0.075M succinate buffer at pH 4.2 resulted in an increase in absorbance at 628 nm. The absorbance/concentration relationship was stated to be linear for samples containing up to 60.0 g/l albumin. Bilirubin, moderate lipaemia and salicylate did not interfere with the analysis. The use of a nonionic surfactant (Brij-35) reduces the absorbance of the blank, prevents turbidity and provides linearity. The authors found that results obtained using their method agreed favourably with those obtained by electrophoresis and salt fractionation. The authors main criticism of the Rodkey (1965) method was the very high absorbance of the working dye solution which required the use of a sensitive and precise photometer.

Pemberton and De Jong (1971) investigated the relative dye binding capacity of albumin from several species with B.C.G., H.A.B.A. and Spectru AB-2 dyes and compared the results with those by a cellulose acetate electrophoretic technique. Witiak and Whitehouse (1969) had already reported that there was as much as a tenfold difference in absorbance between bovine and equine

albumins using the H.A.B.A. procedure and Pemberton and De Jong presented data which supported these observations. The authors showed that statistically the B.C.G. method and the electrophoretic method provided comparable results, but like the H.A.B.A. dye, Spectru AB-2 also showed wide differences in relative absorbance values when reacted with albumin from various species. It was recommended that the B.C.G. procedure be adopted with species specific standards when the albumin content from several species is to be determined.

Ferreria and Price (1974) compared an automated B.C.G. and immunoprecipitin (I.P.) method for the determination of serum albumin. It was shown that the B.C.G. method gave serum albumin levels higher than the I.P. method by an order of 5.0 g/l but these differences were less with icteric sera. It was suggested that the most likely explanation for the discrepancy in results was that B.C.G. is not entirely specific in its binding to albumin. Non specific reactivity of B.C.G. with other serum components gives falsely high results and this non specific binding was estimated to be approximately 15% of the total. It was suggested that the reason for the decreased difference in results between the B.C.G. and I.P. methods with icteric sera was that bilirubin competes with B.C.G. for similar binding sites on the albumin molecule.

Webster, Bignell and Attwood (1974) compared human serum albumin levels determined by an automated B.C.G. method, a cellulose acetate (C.A.) method and two I.P. methods, the Mancini radial immunodiffusion and the Laurell 'rocket' immunoelectrophoresis techniques. The author found that the C.A. method gave albumin results in good agreement with the I.P. methods but the B.C.G. method tended to give high

results at low albumin values and low results at high albumin values when compared with the C.A. method. Webster (1974) conducted experiments to investigate the specificity of B.C.G. for albumin using a continuous flow automated method and found that the alpha and beta globulin fractions in human serum reacted with the dye reagent producing significant 'albumin' peaks. The gamma globulin fraction of serum did not react with B.C.G. From his experiments the author found that both the alpha and beta globulins do not react weight for weight with B.C.G. as does albumin but only produce about one-fifth and one-tenth of the colour intensity respectively that a comparable weight of albumin produces. The author concluded that results from his study cast considerable doubts over the specificity of B.C.G. for albumin and explains the discrepancy in results between the B.C.G. method and the C.A. method in the determination of low albumin levels in abnormal sera.

Gustafsson (1976) proposed a means of improving the specificity of serum albumin determination by the B.C.G. reaction and also to estimate the 'acute phase reactants'. The author postulated that the reaction of serum with B.C.G. proceeds in two steps with albumin being responsible for the faster (< 1 min) reaction. The slower (30 min) reaction is a measure of the 'acute phase reactants' in serum (proteins such as orosomucoid and caeruloplasmin are accepted as being acute phase reactants.)

Webster (1977) investigated the 'immediate' (< 30 secs) reaction between bromocresol green and serum as a measure of albumin content. Fifty specimens of human serum were analysed in duplicate for albumin by C.A. electrophoresis using a procedure

previously shown to give results in good agreement with two immunoprecipitin techniques (Webster et al, 1974) and by a dye-binding method with the absorbance being read as soon as possible after mixing. Results by the dye binding method were found to be 3.0 g/l higher than those obtained by electrophoresis; this difference was independent of the albumin content of the serum. Webster suggested that apparently the immediate reaction between serum and dye provided a simple reliable measure of albumin content when 3.0 g/l was subtracted from the results obtained. He also pointed out that automated continuous flow systems with various combinations of mixing coils would give erroneously high results, the magnitude of which depends on the length of time between mixing the sample with B.C.G. reagent and measurement of the absorbance. The same applies to any manual B.C.G. methods which include a colour development period. Owing to the time dependent interference between B.C.G. and non-albumin reacting material, it was recommended that the time interval between colour development and absorbance measurement should be kept to the absolute minimum to ensure results that more nearly represent the true albumin concentrations as determined by more specific techniques.

In an attempt to overcome the problems of erroneously high serum albumin values with automated B.C.G. dye methods as pointed out by Webster (1977), Gustafsson (1978) devised a simple and rapid method of reliably determining albumin automatically. He showed that the 'immediate reaction' for albumin could be automated and described the application of the B.C.G. dye technique in both the automated reaction rate analyser and multichannel analysis system.

More concentrated reagents than those described by Doumas et al. (1971) were used, with the concentration of the B.C.G. dye reagent in the former system being fivefold that of Doumas and in the latter system threefold the concentration. Using the reaction rate analyser it was possible to extrapolate back to the exact moment of adding serum to the working dye solution, to obtain the most nearly accurate albumin results. This could be done either manually or automatically by a computer and the same was also possible when using the multichannel analysis system. In this way any possible errors due to interferences previously described were kept to the absolute minimum. The author also found that acceptable results were obtained when reaction times of 8-10 secs. were used. Corcoran and Durnan (1977) also found this to be a acceptable limit. An important factor in the automated variations of the B.C.G. dye method is a predilution step of serum with distilled water. This was preferred to other diluents such as sodium chloride at 9.0 g/l concentration or the succinate buffer used in the preparation of the B.C.G. dye solution, as they gave erroneously high albumin values. The author suggested that this was due to a conformational rearrangement of one or more of the slow reacting proteins in the diluted samples; the rearranged protein reacting immediately with B.C.G. as if it were albumin.

Although never developed for the clinical determination of serum or plasma albumin, solutions of some other dyes have shown spectral shifts in the presence of albumin and have been used in various experiments involving albumin. Huggins, Jensen, Player and Hospelhorn, (1949) studied the binding of phenolsulphonphthalein by serum albumin and Brenner (1952) observed the effects of combining

trypan blue, Evans blue and Congo red dyes with the plasma albumin from some animal species. Later Waldmann-Myer and Schilling (1956) investigated the interaction of bromophenol blue with albumin and gamma globulin in acid medium.

Recently interest has been revived in the use of bromocresol purple (B.C.P.) dye as a means of determining serum albumin concentration (Pinnell and Northam, 1978). Previously Louderback, Mealy and Taylor (1968) had developed a B.C.P. dye binding technique for the determination of albumin in serum which was modified by Carter (1970), but little attention had been paid to the possibilities of the technique during the intervening period. This apparent lack of interest may have been partly due to the reasons described by Pemberton and De Jong (1971) in their comparison of the relative dye binding capacities of albumin from several species with H.A.B.A., B.C.G. and Spectru AB-2. Pinnell and Northam (1978) describe B.C.P. as an indicator dye similar in structure to B.C.G. and they detail a new automated B.C.P. method which they state to have several advantages over the existing B.C.G. methods while still retaining the desirable properties of the latter. Unlike B.C.G., B.C.P. did not react with albumin-free globulin preparations or with pure human transferrin solutions and reaction with serum was instantaneous, in contrast with B.C.G. which exhibits a time dependent reaction as indicated previously. Comparison between the B.C.P. and B.C.G. methods and electroimmunoassay showed that albumin results using the B.C.P. method agreed well with the latter, but those obtained by the B.C.G. were found to be considerably higher. The authors noted that sodium salicylate added to serum did not interfere with the B.C.P. method up to at least 300 mg/l, but there

was some interference from sera which were haemolysed, lipaemic or icteric. Blank correction was made for grossly haemolysed sera (approximately 1g. of apparent albumin per litre at 10g. of haemoglobin per litre). Grossly lipaemic sera required the greatest correction (up to 3.5 g/l), in extreme cases. The authors acknowledged that the use of albumin from various animal species was not suitable for calibration or control of the B.C.P. method for determining human albumin. They found the reactivity of equine and bovine albumin to be much less than that of human albumin.

Rudolph and Venegas (1979) found the B.C.P. method unsatisfactory when estimating equine serum albumin as they reported a poor correlation of results obtained by the B.C.P. method and by paper electrophoresis; with the mean albumin levels by the B.C.P. method being 6.0 g/l higher than those by electrophoresis.

Keay (1980) (unpublished observation) noted that although there was a linear absorbance/concentration relationship up to 60 g/l between B.C.P. and human, equine bovine and ovine purified albumin at 603 nm., there was a wide variation in relative binding capacity between species, with bovine and ovine albumin in particular having very low absorbance values when combined with B.C.P. even at albumin concentrations of 60.0 g/l.

Although the B.C.P. method of Pinnell and Northam does seem generally favourable for the determination of human albumin in serum or plasma, it does not seem to be a suitable alternative to the existing B.C.G. methods for determining serum or plasma albumin from animal species in spite of its apparent greater specificity.

CHAPTER IV

REVIEW OF THE LITERATURE

RESULTS OBTAINED IN CATTLE SHEEP AND HORSESALBUMIN BY DYE-BINDING TECHNIQUES

Pemberton and de Jong (1971) compared various automated procedures for the analysis of serum albumin with a view to finding one which would be best suited for use with several species. The relative dye binding capacity of three different dyes was studied with serum albumin from cattle, sheep, horses and some other species. Results using the dye binding methods were compared with those for albumin obtained by cellulose acetate electrophoresis.

It was found that figures obtained using a B.C.G. method (Northam and Widdowson, 1967) were in better overall agreement with the electrophoretic results for each species than those by the H.A.B.A. (Witiak and Whitehouse, 1969) and Spectru AB-2 (Gindler and Hanson, 1969) methods.

The authors quoted figures which gave mean albumin values of 29.8 g/l, 33.5 g/l and 52.5 g/l for bovine, equine and ovine sera respectively using the B.C.G. method. The same sera gave mean values of 31.4 g/l, 38.8 g/l and 50.6 g/l respectively with cellulose acetate electrophoresis. The mean albumin values for the same sera using the H.A.B.A. method were 21.7 g/l, 40.1 g/l and 48.7 g/l respectively and 17.7 g/l, 37.3 g/l and 48.0 g/l using the Spectru AB-2 method.

Sykes and Field (1973) studied the levels of total protein, albumin and some other biochemical parameters in the serum of Scottish blackface ewes. The effects of protein deficiency during pregnancy on these levels was monitored. Albumin was measured by the H.A.B.A. method of Ness, Dickerson and Pastewka (1965) and total protein by the method of Weichselbaum (1946). (For selected results

see table page 45).

Pierce (1975) also used the H.A.B.A. method of Ness, Dickerson and Pastewka (1965) to analyse the albumin concentration of various equine sera and compared the results with these obtained using cellulose acetate electrophoresis. Two sets of albumin concentrations were obtained using the dye binding method. One set was obtained from a standard curve prepared with standardised human protein (H.A.B.A. dye method 'A') and the other from a standard curve of standardised equine protein (H.A.B.A. dye method 'B'). Low values for equine albumin concentration were obtained using method 'A' ($\bar{x} = 12.9$ g/l) and although higher results were obtained with method 'B' ($\bar{x} = 18.8$ g/l) both sets were substantially lower than the mean value of 27.6 g/l obtained by electrophoresis.

Liberg (1977a) used the B.C.G. method of Doumas, Watson and Biggs (1971) in his chemical and electrophoretic study of serum proteins in clinically healthy cows. Figures were also tabulated to illustrate the seasonal variation of the serum protein pattern in healthy dairy cows. Total protein was determined by the biuret method. (Albumin figures see table page 45). The same B.C.G. method was also used by Liberg (1977b) in a further study of the serum proteins in adult cattle with different diseases.

Khalaf (1977) studied the albumin levels and other biochemical parameters of ewes at lambing. The ewes were classified into high and low nutritional groups and also according to litter size i.e. single lambs, twins and triplets. The B.C.G. method of Doumas, Watson and Biggs (1971) was used to obtain the albumin concentrations and the mean values for ewes in the high nutritional group for single lambs, twins and triplets were $27.7 \text{ g/l} \pm 1.4$,

TABLE 4.1

SOME NORMAL SERUM ALBUMIN LEVELS IN CATTLE SHEEP AND HORSES BY
DYE BINDING METHODS.

AUTHOR	SPECIES AND NUMBER OF ANIMALS	MEAN ALBUMIN CONCENTRATION g/l
Sykes and Field (1973)	S.B.F. Sheep 4th week of pregnancy (28)	31.9 (a)
Sykes and Field (1973)	S.B.F. Sheep 20th week of pregnancy (28)	23.0 (a)
Dargie & Berry (1979)	S.B.F. Sheep (12)	35.0 (a)
Liberg (1977a)	Dairy Cattle (25)	40.5 (b)
Mason and Kwok (1977)	T.B. Race Horses (Young) (106)	36.5 ± 2.4 s.(b)
Mason and Kwok (1977)	T.B. Race Horses (Old) (111)	36.1 ± 2.2 s.(b)
Matthews (1981)	Horses and Ponies (48)	28.9 ± 3.8 s.(b)

(a) H.A.B.A. Dye Method.

(b) B.C.G. Dye Method.

s. Standard deviation.

TABLE 4.2a

NORMAL BOVINE SERUM PROTEIN ELECTROPHORESIS - SOME AUTHORS QUOTING PERCENTAGE (%) FIGURES.

Source	Irfan (1967) Cows over 2 y.o. (100) Paper		Ek (1969) Cows over 2½ y.o. (50) Paper	
	\bar{x}	s	\bar{x}	s
Albumin	43.1		47.4	4.5
Globulins				
Alpha	11.0		11.6	1.3
Beta	12.0		12.1	1.5
Gamma	33.9		28.9	5.1

Statistical definitions and abreviations.

\bar{x} = mean

se = standard error

s = standard deviation

CAM = Cellulose acetate membrane

TABLE 4.2b

NORMAL BOVINE SERUM PROTEIN ELECTROPHORESIS - SOME AUTHORS QUOTING ABSOLUTE (g/l) FIGURES.

Source	Osbaldiston (1972) Cows (92) CAM			Tumbleson et al. (1973) Cows (61) 1½-2½ y.o. CAM			Tumbleson et al. (1973) Cows (28) 3½-4½ y.o. CAM			Liberg (1977a) Dairy Cows (25) Agarose			Liberg (1977a) Stall fed Cows (25) Agarose			James and Polan (1978) Holstein Calves (27) CAM		
	\bar{x}	s		\bar{x}	se		\bar{x}	se		\bar{x}	s		\bar{x}	s		\bar{x}	s	
Fraction																		
Total Protein	70.0			81.4	0.6		87.3	1.2		76.6	4.3		75.3	3.5		64.5	13.4	
Albumin	33.0			42.0	0.6		41.7	0.7								27.0	5.5	
Globulins																		
Alpha																16.2	3.9	
Alpha-1	9.0			11.2	0.4		11.8	0.6		2.2	0.4		2.6	0.5				
Alpha-2										4.6	0.5		4.7	0.4				
Inter Alpha/Beta										1.6	0.4		4.8	1.7				
Beta				8.6	0.2		8.5	1.1								18.9	2.3	
Beta-1	7.0									7.9	0.8		7.4	0.8				
Beta-2	16.0									6.8	1.0		6.2	1.1				
Gamma	12.0			19.7	0.6		25.3	1.1		13.0	2.4		12.6	2.5		12.4	3.9	

For statistical definitions see page 46.

TABLE 4.3a

NORMAL OVINE SERUM PROTEIN ELECTROPHORESIS - SOME AUTHORS QUOTING PERCENTAGE (%) FIGURES.

Source	Perk and Lobl (1960)		Perk and Lobl (1960)		Irfan (1967)		Ek (1970)	
	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s
Fraction								
Albumin	41.7	2.1	56.8		61.7	3.1	48.5	3.2
Globulins								
Alpha-1	3.1	0.3	6.5		2.5	0.4	8.1	1.3
Alpha-2	4.0	0.3	8.8		3.5	0.5	12.6	1.7
Alpha-3	7.5	0.6			10.7	1.6		
Beta	7.5	0.5	9.1				8.0	1.5
Beta-1					5.6	0.9		
Beta-3					4.0	0.3		
Gamma-1	25.5	1.9	18.8		11.6	1.1	22.8	
Gamma-2	10.6	1.3						

For statistical definitions see page 46.

TABLE 4.3b

NORMAL OVINE SERUM PROTEIN ELECTROPHORESIS - SOME AUTHORS QUOTING ABSOLUTE (g/l) FIGURES.

Source	Perk and Lobl (1960)		Perk and Lobl (1960)		Osbaldiston (1972)		Knight and Leek (1973)	
	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	se
Fraction								
Total Protein	73.6		57.8		78.0		69.0	1.5
Albumin	30.7		35.6		36.0		50.1	0.7
Globulins								
Alpha-1	2.3		1.4		3.0		0.9	0.1
Alpha-2	2.9		2.0		4.0		1.4	0.1
Alpha-3	5.5		6.2				2.1	0.2
Beta-1	5.5		3.3		8.0		2.3	0.2
Beta-2					6.5		1.9	0.1
Beta-3	2.3							
Gamma-1	18.8		6.7		16.0		2.0	0.2
Gamma-2	7.8				4.0		6.7	0.6

For statistical definitions see page 46.

TABLE 4.4a

NORMAL EQUINE SERUM PROTEIN ELECTROPHORESIS - SOME AUTHORS QUOTING PERCENTAGE (%) FIGURES.

Source	Kao et al. (1954)		Irfan (1967)		Bierer (1969)		Ek (1970)		Pierce (1975)		Kristensen & Firth (1977)		Rudolph & Venegas (1979)		Rose & Paris (1979)	
	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s
Fraction																
Albumin	39.6	1.6	33.5		43.0		47.0	4.0	41.5		36.2	4.7	53.2	5.19	39.9	4.8
Globulins																
Alpha-1	16.9	2.0	15.0				4.3	1.1	7.0		4.4	1.2	3.25	0.79		
Alpha-1a					2.2											
Alpha-1b					3.1											
Alpha-2	15.0	3.5	16.0				9.9	1.9	11.6				9.69	2.18	16.6	2.2
Alpha-2a					12.8											
Alpha-2aa											5.0	1.4				
Alpha-2ab											2.6	0.7				
Alpha-2b					5.6						3.5	0.4				
Alpha-2c											3.6	1.1				
Beta	13.6	2.1	15.5		11.9		19.5	2.9	22.6		10.4	2.8				
Beta-1											15.1	3.4	10.18	3.44	22.3	3.3
Beta-2a					3.5											
Beta-2b					3.7											
Gamma-1	14.9	3.3	20.0		12.1		19.0	3.3	17.3		8.2	2.9	7.11	1.95		
Gamma-2					2.2						17.3	4.0	16.82	3.84	21.8	3.1

TABLE 4.4b

NORMAL EQUINE SERUM PROTEIN ELECTROPHORESIS - SOME AUTHORS QUOTING ABSOLUTE (g/l) FIGURES.

Source	Matthews et al. (1966)			Osbaldiston (1972)			Massip and Fumiere (1974)			Kirk et al. (1975)			Liberg et al. (1977)			Kristensen et al. (1977)			Rudolph et al. (1979)		
	\bar{x}	s	CAM	\bar{x}	s	CAM	\bar{x}	se	CAM	\bar{x}	se	CAM	\bar{x}	s	Agarose	\bar{x}	s	Agarose	\bar{x}	s	Paper
Total Protein	67.4	2.8		73.0			61.0	0.7		75.3	1.6		75.6	4.9		66.4	6.4		61.9	4.9	
Albumin	31.4	2.4		30.0			27.6	0.8		27.0	0.6					23.9	2.7		32.9	3.6	
Globulins																					
Alpha				10.0			2.5	0.2		3.0	0.2										
Alpha-1	1.9	0.3											1.6	0.3		2.9	0.8		1.9	0.5	
Alpha-2	6.5	1.3											7.4	1.3					6.0	1.4	
Alpha-2a																3.3	0.9				
Alpha-2aa																1.7	0.4				
Alpha-2ab																2.3	0.3				
Alpha-2b										2.1	0.2					2.4	0.8				
Alpha-2c							1.7	0.1								0.7	0.03				
Beta-1	9.2	3.0					8.1	0.4		8.2	0.8										
Beta-2	5.7	1.1		10.0			8.1	0.4		12.7	1.1		19.6	4.0		10.1	2.8		6.3	2.0	
Gamma	10.4	1.4		8.0			4.7	0.3		8.2	0.6		4.4	0.8		5.5	2.1		4.4	1.3	
				15.0			8.2	0.5		14.1	0.6		9.8	1.8		11.5	2.8		10.4	2.5	

For statistical definitions see page 46.



25.3 g/l \pm 2.9 and 24.8 g/l \pm 2.4 respectively. The mean albumin values for ewes in the low nutritional group with single lambs, twins and triplets were 26.1 g/l \pm 2.4, 24.0 g/l \pm 2.4 and 24.1 g/l \pm 2.3 respectively. (All figures mean \pm SD)

Mason and Kwok (1977) studied some haematological and biochemical parameters in race horses and used the B.C.G. method of Northam and Widdowson (1967) to determine the serum albumin concentration. Total protein was determined by the method of Henry, Sobel and Berkman (1957). (For results from certain groups see table page 45).

Gentry and Lumsden (1978) determined the serum albumin concentration in bovine, equine and certain other species using the immediate B.C.G. reaction as described by Webster (1977). Total protein was determined by the manual biuret method of Weichselbaum (1946). Serum proteins were separated by electrophoresis on agarose gel using a barbital buffer at pH 8.6.

Specific albumin was obtained and standard curves prepared for each species using the B.C.G. method. With each group of serum samples analysed, a bovine albumin standard together with a species specific albumin standard was employed to ensure that results for the standards fell on the standard curve. The figures produced were a mixture of normal and abnormal levels since the material was obtained from both teaching and hospitalised animals.

The authors found that provided the immediate reaction was measured, albumin results using the B.C.G. method with most species compared favourably with the electrophoretic albumin results irrespective of whether bovine albumin or species specific albumin was used to calculate the concentration.

Albumin concentration in pregnant sheep was studied in relation to body protein loss by Sykes and Thomson (1978) using the H.A.B.A. dye method of Ness, Dickerson and Pastewka (1965).

Dargie and Berry (1979) monitored the hypoalbuminaemia of ovine fascioliasis using the H.A.B.A. dye method of Rodkey (1965) to determine the serum albumin concentrations. The sheep were initially divided into two equal groups and fed on a special diet including high protein compounds. After a period equal numbers of sheep were infected with *Fasciola hepatica* metacercariae while the remainder acted as pair fed controls. Over the course of the experiment the albumin levels of the control group remained fairly close to the pre-experiment level (see table page 45) while the levels in the infected group fell to below 20.0 g/l.

Sykes and Russel (1979) studied the seasonal variation in plasma proteins and other parameters in hill sheep. The H.A.B.A. dye method of Ness, Dickerson and Pastewka (1965) was used to determine plasma albumin concentrations. Total protein was estimated by a continuous flow system of Weichselbaum (1946). A total of 414 Scottish blackface ewes were sampled and the authors recorded mean plasma albumin concentrations of 15.5 g/l, 20.7 g/l and 23.5 g/l during winter at three different sites.

Matthews (1981) used the B.C.G. method of Dumas, Watson and Biggs (1971) to study the serum albumin concentration of a group of horses and ponies. (See table page 45). The author compared the mean albumin level obtained by this method with that obtained by agarose gel electrophoresis. The two sets of figures were found to be in good agreement.

PAPER ELECTROPHORESIS

An early application of this technique in the veterinary field was by Boguth (1954) and Kao, Reagan and Brechner (1954). The latter group employed paper electrophoresis in their study of horse serum from cases of equine infectious anaemia. The albumin/globulin ratio obtained using a salt precipitation method was compared with the results obtained by electrophoresis. Equine sera resolved into five peaks, albumin, alpha-1, alpha-2, beta and gamma globulins. Figures for the relative percentages of components from the serum of four normal horses are given (see table page 50).

Perk and Lobl (1960) conducted biuret and paper electrophoretic analysis of normal sheep serum proteins and lipoproteins. The authors found that after electrophoresis, sheep serum gave distinct protein bands of albumin, three alpha globulins and two beta globulins. The gamma-globulin band of sheep corresponded in mobility to that of human, but in the case of adult ewes, rams and older lambs this could be easily subdivided into gamma-1 and gamma-2. (See table page 48). The electrophoretic values obtained for albumin turned out to be consistently lower than those obtained by the chemical method. The authors believed the lower values to be the more accurate since it was clear that unlike the salting out methods, the albumin is obtained completely dissociated from the globulin fractions.

Irfan (1967) conducted a comprehensive study of the patterns of serum proteins in some species of domestic animals using paper electrophoresis. Serum from groups of animals including 100 cattle, 70 horses, and 10 sheep was analysed. Total protein was estimated by a copper sulphate S.G. method. The percentage figures for

albumin, alpha-1, alpha-2, beta and gamma globulins were obtained, except in the case of cattle where the alpha-1 and alpha-2 globulins did not resolve. (See results table page 48).

Rudolph and Venegas (1979) analysed the serum from 60 racehorses electrophoretically and also compared the albumin figures with those obtained by a bromocresol purple dye-binding technique. The figures for electrophoresis are included in Table 4.4b(see page 51).

STARCH GEL ELECTROPHORESIS .

Ashton (1957a) investigated the resolution of serum proteins in cattle, pigs, horses and dogs using the method of Smithies (1955) and concluded that the pattern obtained was a characteristic of the species. Using a modification of the method of Smithies Ashton (1957b) also studied the serum protein differences in several breeds of cattle. Particular attention was paid to the distribution of the beta-2 globulin phenotypes with respect to breed.

Smithies (1959) improved his earlier method and used it to demonstrate over thirty protein components in sera from several species. He concluded that more specific identification of some components was possible using this method and put forward the hypothesis that molecular size played an important role in determining the type of separation obtained.

CELLULOSE ACETATE ELECTROPHORESIS

Matthews, Kaneko, Loy, Cornelius and Wheat (1966) used a commercial microcellulose acetate method in their study of the normal serum protein values in healthy adult horses. (See table

page 51). Total protein was undertaken by a salt fractionation technique.

Bierer (1969) subjected serum and plasma from ten normal horses to electrophoresis on cellulose acetate strips. (See table page 50). The author stated that the main advantages of using cellulose acetate instead of paper were a reduction in analytical time and the fact that more protein bands could be demonstrated. Bierer recorded ten protein bands in equine serum with the alpha-1, alpha-2 and beta-2 globulins being subdivided. Coffman (1969) studied the clinical application of serum protein electrophoresis in the horse using cellulose acetate medium.

Ek (1969, 1970) claimed to be among the first to conduct an experiment using cellulose acetate membrane for the electrophoresis of serum from various domestic animals. (See table page 46). Initially serum from cattle was studied, (Ek 1969) but later serum from normal horses, sheep and pigs was also included (Ek 1970). It was the authors aim to use the technique to obtain the best possible separation and also to further identify the main individual serum protein fractions, by performing electrophoresis on gamma globulin preparations from each species as well as mixtures of gamma globulin and transferrin (a beta globulin component). In general, the authors results were in good agreement with those obtained by Irfan (1967), and Perk and Lobl (1960). Ek (1969) also showed how the addition of calcium ions to a more dilute barbitone buffer could increase the separation between the beta and gamma globulins of bovine serum.

Osbaldiston (1972) reported normal values for serum protein

fractions from various species including cattle, sheep and horses. (See table page 47). The observed distances of migration of the various protein fractions in each species was studied and formulated. Total protein was determined by a biuret method.

Knight and Leek (1973) compared the electrophoretic patterns of bottle raised and ewe raised lambs from birth to 19 weeks of age. In accordance with the nomenclature of Perk and Lobl (1960) the authors also demonstrated 8 serum protein fractions in most of the separations recorded. They however termed the beta-3 peak of Perk and Lobl as beta-2. (See table page 49). Occasionally the alpha, beta and gamma globulins did not separate into subfractions but after one week and for the remaining 18 weeks of the experiment, the gamma globulin separated into its two subfractions. Total protein was determined by an automated method.

In a study which was intended to determine changes in serum protein concentration as a function of age, Tumbleson, Burks and Wingfield (1973) subjected the sera from 311 Guernsey and 199 Holstein dairy cattle to cellulose acetate electrophoresis. Total protein was determined by an automated biuret method and results from certain groups are given. (See table page 47).

Massip and Fumiere (1974) conducted a study of the serum proteins of 98 normal adult horses with ages ranging from four to ten years. (See table page 51). Total protein was determined by a biuret method.

Kirk, Hutcheson and Neate (1975) examined the electrophoretic patterns of serum proteins from 14 clinically normal horses and also ponies with laminitis. (See table page 51). Total protein was estimated by a biuret method and absolute figures calculated.

Pierce (1975) compared some chemical methods for the measurement of albumin, globulin and total protein in sera from clinically normal horses. Total protein was determined by refractometry and by a biuret method and albumin by a dye binding technique. Computations of the concentrations of the individual electrophoretic factors was based on refractometric total protein measurements. (See table page 50).

James and Polan (1978) examined the effects of orally administered duodenal fluid on serum proteins in neonatal Holstein calves by cellulose acetate electrophoresis and figures for albumin, alpha, beta and gamma globulin were reported as well as total protein by a biuret method. Figures for calves 48 hours after birth are included. (See table page 47).

POLYACRYLAMIDE GEL ELECTROPHORESIS

Kawata, Chase, Elijiw and Macek (1971) used the technique of preparative polyacrylamide gel electrophoresis in a protein separation and paid particular attention to horse heart myoglobin. Hyslop (1972) applied an improved system of polyacrylamide gel electrophoresis to studies on the sera of different species. Multilayered gels of 2%-8% were employed and excellent resolution was reported throughout the albumin and globulin regions. Kholod (1974) applied a similar method in his study of the serum proteins of cattle and reported equally favourable resolution. Figures for the relative mobilities of albumin, and certain globulins were tabulated.

Heinert and Klinger (1978) used the technique of polyacrylamide gel electrophoresis as a means of demonstrating the protein differences between animal species. Serum from cattle and pigs was used in

the study but no quantitative results were recorded.

AGAROSE GEL ELECTROPHORESIS

Carlstrom and Liberg (1975) slightly modified the method of Johansson (1972) and utilised it in their study of cattle serum proteins. The authors claimed a better separation over the entire electrophoresis picture especially in the beta region and the main bands demonstrated, commencing at the anode were albumin, alpha-1 globulin, or alpha-2 globulin zone which usually separated into two or more bands; a broad region between the alpha and beta regions which contained a series of faint bands; a broad beta-1 globulin zone which contained several very distinct bands; a beta-2 globulin zone which contained a distinct band when the sample was fresh but which, on lengthy frozen storage divided into two or more diffuse bands; a gamma globulin region which formed a background to the beta-2 zone continued usually as a broad diffuse zone on the cathodal side of the application point.

Liberg and Carlstrom (1976) undertook a study of transferrin polymorphism in Swedish cattle using agarose gel electrophoresis. The polymorphic transferrin patterns from 894 cattle were examined.

Liberg (1977a) used the same method and conditions in his fractionation of serum proteins in clinically healthy dairy cows. The author found that since the albumin zone becomes relatively heavily stained in this electrophoretic procedure, optical measurement using a gel scanner usually underestimates the albumin. For this reason only the relative concentrations of different globulins were taken into account. The absolute concentration was calculated on the basis of total protein minus the albumin concentration as obtained by a biuret and a bromocresol green dye binding technique respectively. (See table page 47).

Liberg (1977b) again used the same electrophoretic method and conditions in his fractionation of serum proteins in cattle with different diseases ranging from displacement of the abomasum to subacute-chronic pneumonia.

Liberg, Magnusson and Schougaard (1977) studied the protein composition of the synovia and serum in healthy horses using the method of Carlstrom and Liberg (1975). Absolute values for alpha-1, alpha-2, beta-1, beta-2 and gamma globulins are presented (see table page 51) as well as albumin values obtained by a bromocresol green dye binding technique.

In the early 1970's commercially prepared agarose gel electrophoresis plates were developed for use in a cassette electrophoresis system and Kristensen and Firth (1977) used the system developed by Corning ACI, Palo Alto, California in their analysis of serum proteins and cerebrospinal fluid in clinically normal horses.

The nomenclature of the bands for serum proteins were presented. (See table page 50). Agarose gel electrophoresis of equine serum resulted in excellent resolution of the six major protein fractions. In addition the so called alpha-2 fraction divided into a maximum of four subdivisions termed alpha-2aa, 2ab, 2b and 2c in many cases. The authors draw attention to the disagreement on the nomenclature of equine serum protein fractions and point out the numerical overlap between subfractions of alpha-1 and alpha-2 globulins. Total protein was determined by a biuret method and both percentage and absolute figures reported.

CHAPTER V

MATERIALS AND METHODS

1. PREPARATION OF STOCK ALBUMIN SOLUTIONS

Three species of albumin Fraction V (Fr. V) were obtained from Miles Laboratories Ltd., Stoke Poges, England and each was supplied accompanied by a purity certificate. Bovine albumin Fr. V was stated to be 98% pure and ovine and equine albumin were both stated to be 99% pure.

Approximately 4g of each species of albumin was weighed into 50 ml glass beakers and reconstituted with approximately 30 ml distilled water containing 0.05% sodium azide as preservative. When reconstitution was complete, the solutions were transferred to 50 ml volumetric flasks and the final volumes made up to the mark with 0.05% aqueous sodium azide.

(a) ANALYSIS OF STOCK ALBUMIN SOLUTIONS

Analysis of the bovine, ovine and equine stock albumin solutions was performed using the biuret technique of Henry, Sobel and Berkman (1957). The protein standard employed was prepared from Pentex[®], bovine albumin, crystalline 10% solution and obtained from Miles Laboratories, Stoke Poges, England. The protein control serum used was Seronorm[®] Batch 144, Nyegaard, Oslo, Norway. (U.K. agents B.D.H. Chemicals, Poole, Dorset, England). The following were set up in separate 125 x 16 mm glass test tubes:-

REAGENT BLANK,	5 ml of 0.75 M NaOH (750 m mol/l).
STANDARD,	4.9 ml of 0.75 M NaOH plus 100 μ l protein standard.
CONTROL,	4.9 ml of 0.75 M NaOH plus 100 μ l protein control serum.
UNKNOWN,	4.9 ml 0.75 M NaOH plus 100 μ l specimen.

To each of the tubes 1 ml of biuret reagent was added, mixed and incubated at room temperature for 15 minutes. The final absorbances of the protein/biuret complexes were read at 545 nm against the reagent blank in 1 cm plastic cuvettes in a Cecil CE 292 U.V./visible range digital spectrophotometer (Cecil Instruments Ltd., Milton Industrial Estate, Cambridge, England). The protein concentration of each albumin solution and control was calculated from the absorbances as follows:-

$$\frac{A \text{ UNKNOWN}}{A \text{ STANDARD}} \times \text{protein conc. of standard} = \text{g/l protein.}$$

The three stock albumin solutions were further checked for protein concentration by a Micro-Kjeldahl technique (see Kerr acknowledgements) and results by the two different methods were as follows:-

	Biuret analysis (mean of 8 replicates) (g/l)	Micro-Kjeldahl (g/l)
Bovine albumin stock soln.	75.0	75.2
Ovine albumin stock soln.	70.2	70.7
Equine albumin stock soln.	74.8	75.2

(b) PREPARATION OF WORKING ALBUMIN STANDARDS

From the stock albumin solutions whose concentrations were established in (a) by the biuret method, a series of six working albumin standards were prepared for each species to cover the range 10 - 60 g/l. The individual volumes required for dilution were calculated assisted by the simple formula $\frac{R \times V}{O} = \text{ml required.}$

where R = required concentration in g/l

V = total volume required in ml

O = original concentration in g/l.

Appropriate volumes were thus calculated and the aliquots of stock albumin solutions were accurately pipetted into 10 ml volumetric flasks; the final volumes being made up with 0.05% sodium azide followed by thorough mixing. The use of sodium azide as preservative allows the working standards to be kept at 4°C for several months without deterioration.

(c) ANALYSIS OF WORKING ALBUMIN STANDARDS

By biuret method

Each of the three sets of working albumin standards were analysed in duplicate for protein content by the technique described in (a) and the actual concentrations of each individual standard were recorded.

By bromocresol green dye-binding technique

The method used was that of Doumas, Watson and Biggs (1971). Stock bromocresol green solution 0.60 mM and the 0.1 M succinate buffer pH 4.00, necessary to prepare the working dye solution was obtained from B.D.H. Chemicals, Poole, Dorset, England as was Brij-35 which was prepared as a 30% solution and added to the working dye solution in a strength of 4.0 g/l. The working dye solution was prepared in a strength of 0.15 mM as recommended by the authors.

The absorbance peak of each species of albumin was determined by reacting 25 μ l of each 30.0 g/l working albumin standard with 5 ml (150 μ mol/l) of working dye solution for 10 mins. in 125 x 16 mm glass test tubes. Absorbances were read in plastic cuvettes against a blank of working dye solution in a Cecil CE 292 digital spectrophotometer and the absorbance peaks of the bovine, ovine and equine albumin solutions were found to be 628 nm.

(d) PREPARATION OF ABSORBANCE/CONCENTRATION CALIBRATION CURVES
FOR EACH SPECIES OF ALBUMIN BY BROMOCRESOL GREEN DYE-
BINDING TECHNIQUE.

The full range of working standards for each species was analysed in the same way as (c), covering the range 10-60 g/l respectively. The absorbance of each standard was read at 628 nm and a calibration curve constructed for each species of albumin. For comparison, a similar series of human working albumin solutions was prepared and analysed in exactly the same way as the three animal species (See Fig. 5.1). A linear relationship between absorbance and concentration was demonstrated over the range 10-60 g/l albumin for the species analysed.

2. AGAROSE GEL ELECTROPHORESIS OF SERUM PROTEINS

The system used was the Corning Agarose Film/Cassette Electrophoresis System, Palo Alto, California, U.S.A. (See Fig. 5.4).

The buffer used was 0.05 M Sodium barbital with 0.035% ethylene-diamine-tetra acetic-acid (E.D.T.A.). This was supplied in premeasured vials (Corning Cat. No. 470. 18. 000) with sufficient to prepare 6 litres of buffer in each pack. The pH was adjusted with 2N HCl to 8.6 using a Beckman 3500 digital pH meter. (Beckman Instruments Inc., Fullerton California, U.S.A.)

Agarose film used in conjunction with the barbital/E.D.T.A. buffer was supplied in sealed metal foil envelopes (Corning Cat. No. 470. 10. 400). Immediately before use the agarose film was peeled from its protective plastic backing before samples were applied with a Corning ACl Quantitative Microlitre Sample Dispenser (Cat. No. 470152) with disposable plastic tips (Cat. No. 470154).

As recommended by the manufacturers, electrophoresis was carried out at 90 volts for 35 mins in barbital/E.D.T.A. buffer at pH 8.6. The electrophoresis power pack used was a Vokam 2541 Power Supply (Shandon Scientific Co. Ltd., Pound Lane, Willesden, London).

Staining of serum protein bands was carried out using 0.2% Amido Black 10 B stain which was supplied in pre measured vials (Corning Cat. No. 470. 12. 000) for preparation in 5% acetic acid. After clearing in 5% acetic acid the stained films were dried between sheets of special filter paper (Corning Cat. No. 470158) in an incubator at 60°C. Densitometric scanning of the stained, dried films was carried out on a Beckman R112 Scanning Densitometer (Beckman Instruments Ltd., Fullerton, California, U.S.A.)

(a) DENSITOMETRIC TRACE OF SERUM PROTEIN ELECTROPHORESIS PATTERNS -
METHOD OF RECORDING AND ANNOTATION.

Using the materials described in 2., serum was subjected to agarose gel electrophoresis.

0.8 μ l of each serum was applied to six of the eight sample slits (S) situated on the cathodal (-) side of an agarose film (See Fig. 5.2) which was then positioned in the cassette cover (a) (See Fig. 5.4). 95 ml of buffer was measured into each of the two compartments of the electrophoresis cassette (b) (See Fig. 5.4) and the cover located in position. The cassette was connected to the power supply and electrophoresis commenced.

After electrophoresis the film was submerged face upwards in a plastic dish and stained for 15 minutes. After draining off excess stain and washing for 2 minutes in 5% acetic acid, the film was then dried for 20 minutes as described in 2.

The dried film was again washed in 5% acetic acid to remove any excess stain and further dried for a few minutes. After this treatment the background appeared perfectly clear, showing several well defined blue stained protein bands. (See Figs. 5.2 and 5.3)

The stained film was then scanned in the densitometer which produced a pen trace of the density of each protein band together with an integral of the density trace. This is shown as a zig-zag line below each protein band. The number of times this line traverses the set of 10 horizontal lines within its scale is proportional to the density of each individual protein band. (See Fig. 5.5)

To calculate the density count of each protein band, vertical lines were drawn through the mid-point of the valleys between each peak of the density trace and projected downwards to a point where this line, as well as a zig-zag and horizontal lines coincided. (See Fig. 5.5). The number of times the zig-zag line traversed the horizontal scale between each pair of projected vertical lines was thus calculated for each band, each full traverse being counted as 10. The density counts for each individual band were totalled and expressed as a relative percentage.

(b) CALCULATION OF R_f VALUES.

To calculate the relative fraction (R_f) values of each protein band from the densitometer trace (i.e. relative distance each band has migrated) distances were measured and related to the fastest migrating band which is taken as 1.0. The sample application point which appeared as the first small indentation on the cathodal side of the pen trace was taken as zero or the reference point for

FIGURE 5.1

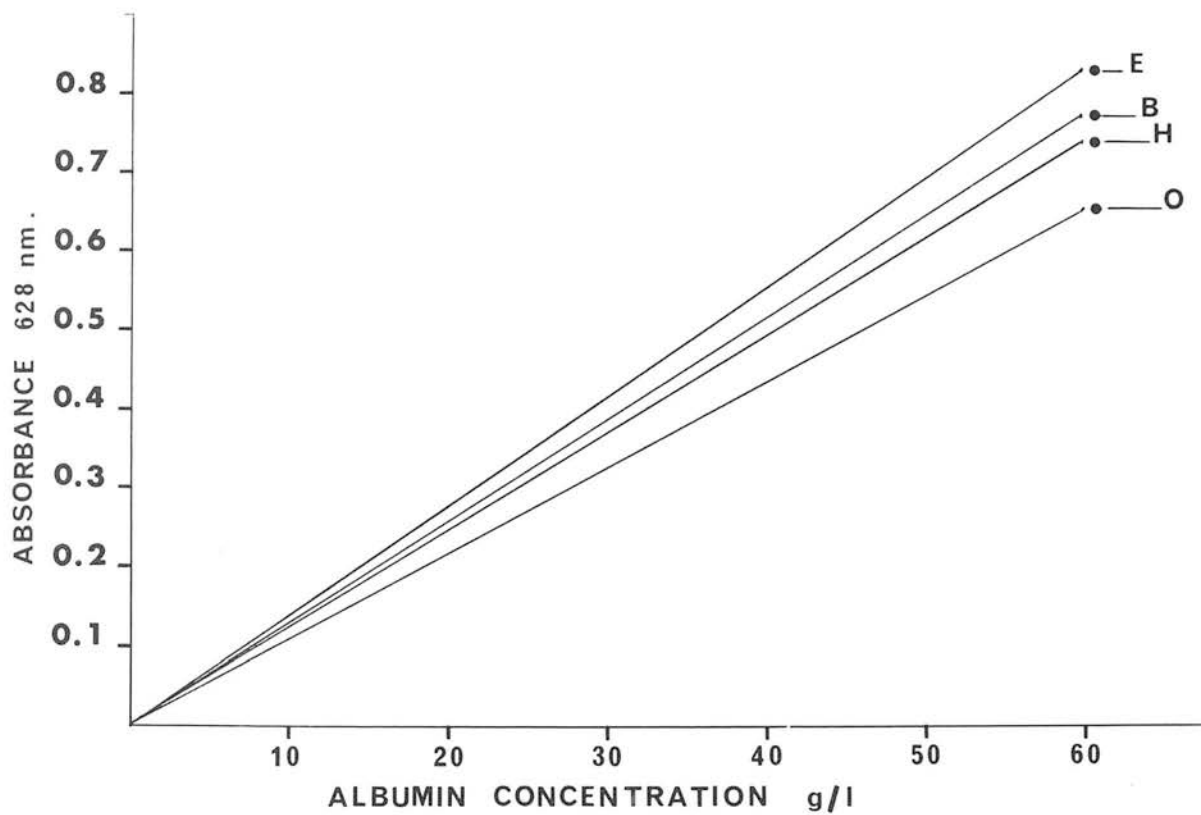
ABSORBANCE-CONCENTRATION RELATIONSHIP OF FOUR
SPECIES OF ALBUMIN WITH 0.15 mM BROMOCRESOL
GREEN (B.C.G.) WORKING DYE SOLUTION.

E = EQUINE

B = BOVINE

H = HUMAN

O = OVINE



measurement (See Fig. 5.6). Vertical lines were drawn from the mid point of each peak on the trace and projected down to a line joining the reference point and the maximum Rf of 1.0. The distances of each peak from the reference point were thus measured e.g. in millimetres and expressed as a fraction of 1.0.

3. 'ROCKET' IMMUNOELECTROPHORESIS

'Rocket' Immunelectrophoresis (I.E.P.) of serum albumin was carried out in a Shandon Universal Electrophoresis Tank (Shandon Scientific Instruments, London) using a tris/barbital high resolution buffer pH 8.6 and ionic strength 0.02, obtained in pre-measured vials (Gelman Hawksley Ltd., Lancing, Sussex).

Anti-bovine serum albumin for the preparation of 1% agarose gel containing antibodies was obtained from Miles-Yeda Ltd., Stoke Poges, England and both anti-equine and anti-ovine albumin of concentration approximately 2.4 - 3.0 mg/ml from Nordic Pharmaceuticals (U.K. agents Fraburg Ltd., Furze Platt Road, Maidenhead, England). Agarose was obtained from Sigma Chemical Company Ltd., Fancy Road, Poole, England.

Stock agarose 2% strength in buffer was prepared and stored in 5 ml aliquots in glass universal containers at 4°C.

Prior to preparing an agarose plate, a 5 ml aliquot of 2% agarose was melted in a hot water bath at 80°C and after cooling slightly, mixed with an equal quantity of buffer containing 100 µl of appropriate antisera. When a temperature of 56°C was reached the 1% agarose gel containing antibodies was carefully poured on to an 8 cm glass plate resting on a level surface. The gel was then allowed to congeal at 4°C in a moist chamber. Gel adhesion

was promoted by coating the plate with a fine layer of 1% agarose which was dried in an incubator prior to the gels being poured. After 2 hours, 12 x 3 mm diameter sample wells, suitable for 5 μ l of sample were cut in the 1.5 mm thick gel using a template and gel punch and the cut portions were removed by suction. The wells were positioned in a row 1.5 cm from the base of the plate (cathodal side and 1 cm in from the edges. The plate was in contact with the tank buffer solution by means of filter paper wicks of a similar width to the plate.

The power supply used was identical to that described in 2, but at a voltage of 280 volts.

After electrophoresis the rocket shaped precipitates were stained with 0.5% Amido Black 10 B stain made up in 45 ml ethyl alcohol, 45 ml acetic acid and 10 ml distilled water. Staining was completed in 15 minutes and after washing in 5% acetic acid to remove excess stain a few sheets of fine filter papers were laid on the gel surface. A weight of approx. 1 kg. (e.g. heavy book) was placed on top of the filter paper to assist in compression. After 15 minutes, the partially dried and compressed gel was placed in an incubator at 60°C to dry completely.

(a) MEASUREMENT OF 'ROCKET' PEAK HEIGHTS

Measurement of the peak heights of the rocket shaped stained precipitates was carried out by first placing the plate face upwards on a piece of blue coloured graph paper of scale 1, 5 and 10 mm. Since the background of the plate was almost completely clear the ruled lines were easily visible. The sample application wells were aligned over a suitable bold horizontal line of the paper and the

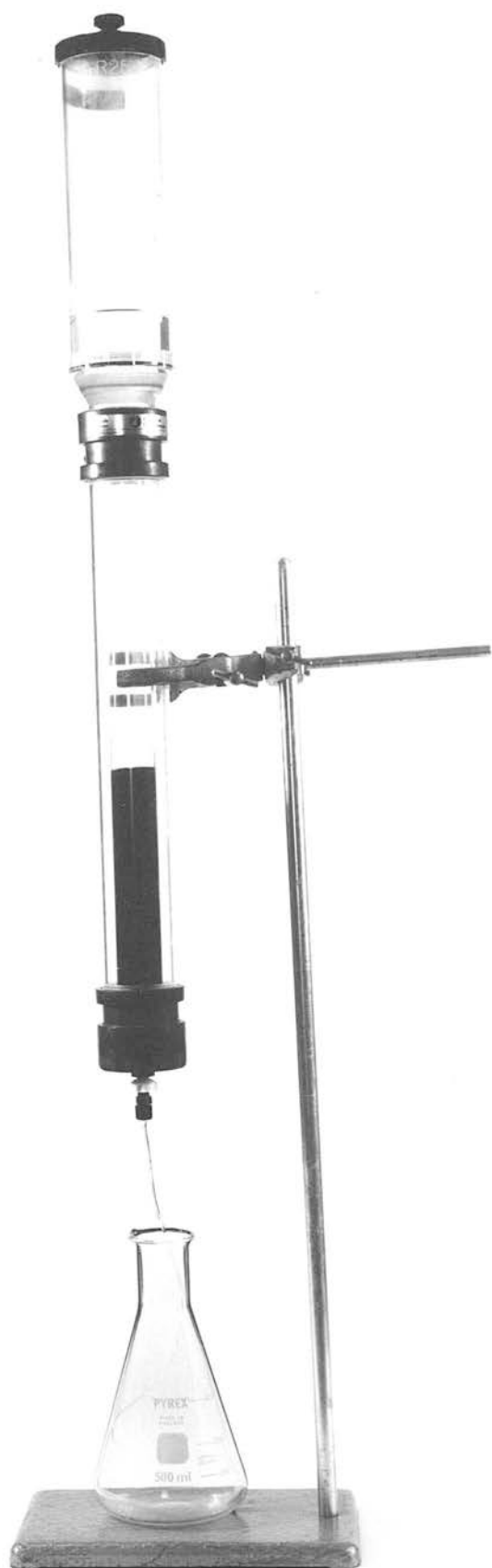


FIGURE 5.8

AFFINITY CHROMATOGRAPHY COLUMN

Containing Affi-gel Blue in lower part of column
and phosphate buffered saline (P.B.S.) in upper part of
column.

peak heights were read off in mm above the base line.

4. AFFINITY CHROMATOGRAPHY. THE PREPARATION OF ALBUMIN FREE SERUM.

(a) REAGENTS AND APPARATUS

Affi-gel Blue 100 ml of 50-100 mesh. (Bio-Rad Laboratories Ltd., Caxton Way, Watford, England).

Phosphate buffered saline (P.B.S.) pH 7.20 (Oxoid Dulbecco 'A', Oxoid Ltd., Wade Road, Basingstoke, Hampshire, England).
1 tablet dissolved per 100 ml distilled water and 0.05 g sodium azide (0.05%) added. De aerated by negative pressure for 15 mins.

1.4 M NaCl. 81.8 g NaCl dissolved in 1 litre P.B.S.

8 M Urea. 560.5 g Urea dissolved in 1 litre P.B.S.

Polyethylene Glycol (P.E.G.) 40%. 400 g P.E.G. dissolved in 1 litre distilled water.

(NaCl, Urea and P.E.G. obtained from B.D.H. Chemicals Ltd., Poole, England).

Dialysis tubing, Visking 24/32. (Scientific Instruments Centre Ltd., Leeke Street, London.)

Chromatography column 40 cm x 2.5 cm (Pharmacia G.B. Ltd., Prince Regent Road Hounslow, England).

Immersible Molecular Separation Kit. (Millipore Corporation, Abbey Road, London.)

(b) PREPARATION OF COLUMN

An affinity chromatography column of Affi-gel blue was prepared with a total bed volume of 5 ml per ml of serum to be

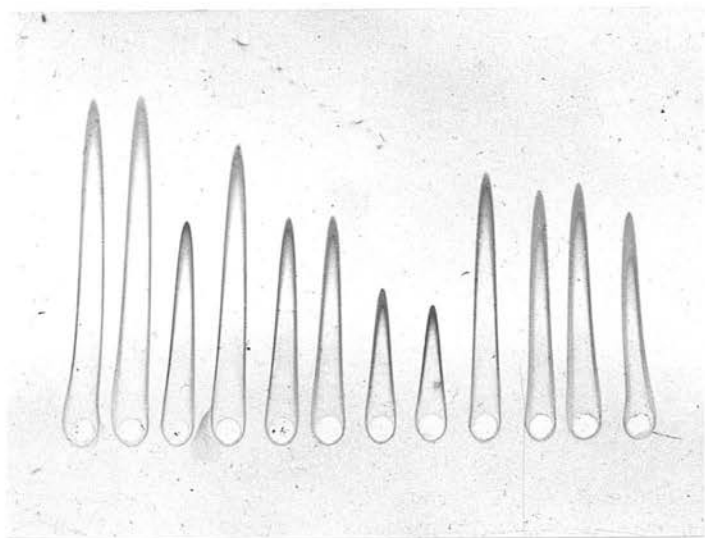


FIGURE 5.7

'ROCKET' I.E.P. PLATE

Positions 1-8 left to right on plate -

bovine albumin Fr.V standards, range 39.5 g/l -

12.5 g/l.

Positions 9-12 - bovine serum albumin.

processed. Since no more than 20 ml of serum from each species, was required to be processed, 100 ml bed volume of Affi-gel blue was used. This was suspended in P.B.S. and allowed to settle in the column by gravity at room temperature for approximately 2 hours, until no more packing was observed. The clear layer of P.B.S. was allowed to run off slowly until the level was just above the top of the gel layer. The column was then washed with two bed volumes of P.B.S. at a rate of 2ml/min (See Fig. 5.8). Care was taken not to allow any part of the gel column to become dry.

(c) PROCESSING SERUM

Serum from each species was individually dialysed against P.B.S. for 12 hours. 20 ml of dialysed serum was applied to the column and washed through with 2 bed volumes of P.B.S. at a rate of 1 ml/min. The effluent was collected, re-applied to the column in the same way and the second effluent was collected and concentrated to approximately 60 ml volume in Visking dialysis tubing against 40% Polyethylene Glycol.

(d) FINAL CONCENTRATION OF ALBUMIN-FREE SERUM

The partially concentrated effluent was further concentrated to a strength of approximately 33 g/l total protein (i.e. globulin) using a set of Immersible Molecular Separators (Millipore (U.K.) Ltd., Abbey Road, London. Cat. No. PTGCOOLK1). These are vacuum-operated filter units for separating macromolecules on the basis of size, in small to intermediate volumes of fluids. Each unit consists of 11 cm² of Pellicon[®] molecular filtration membrane permanently sealed to a cylindrical plastic cone. The retentive surface of the membrane faces outward and the unit is lowered into the solution to be processed before being connected to a vacuum source. Filtrate

passes through the membrane into the cone and then flows via the vacuum line to a suitable trap, the retained molecules remaining in the sample vessel (Millipore, 1975). The total protein was checked at intervals using the biuret method described earlier in 1(a) until the desired concentration was reached (i.e. the approximate globulin concentration in normal whole serum).

The concentrated globulin preparation was subjected to agarose gel electrophoresis by the method described earlier in 2, to check for the presence of any trace of albumin.

(e) COLUMN ELUTION AND REGENERATION

To prepare the column ready for use with another batch of serum the albumin bound to the gel was first eluted with 2 bed volumes 1.4 M NaCl at a rate of 2 ml/min. Regeneration was then completed by running 2 bed volumes of 8 M Urea in P.B.S. through the column at a rate of 2 ml/min.

Bovine serum protein recording and annotation.

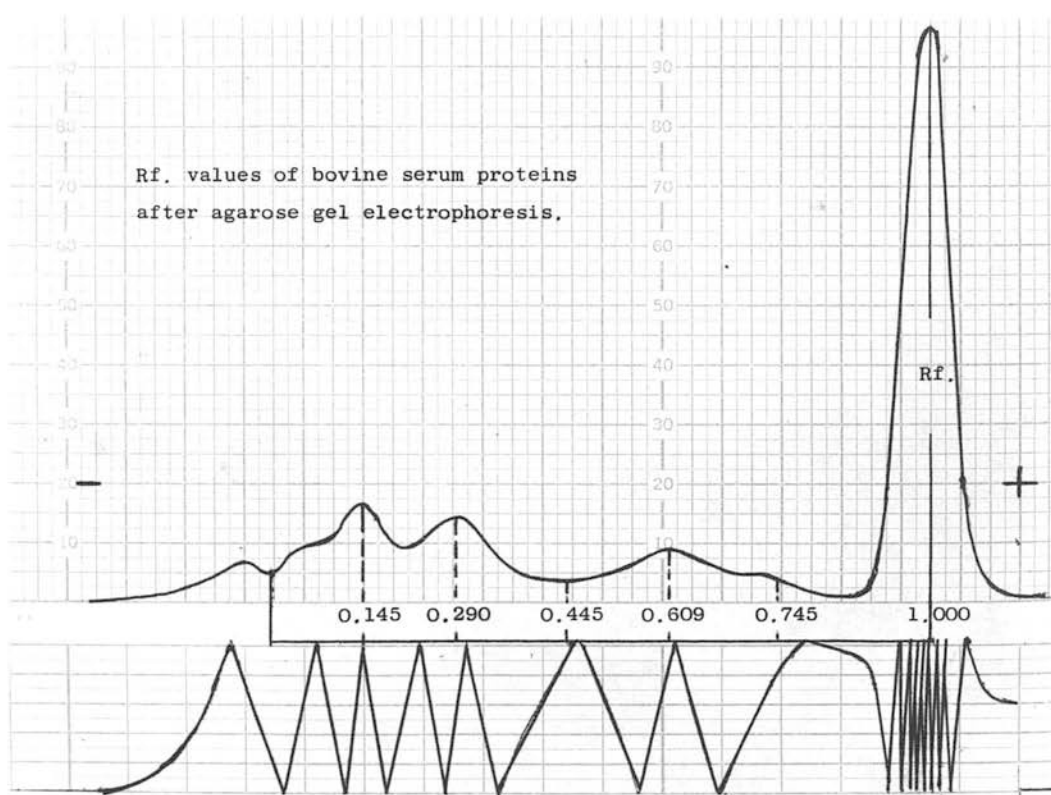
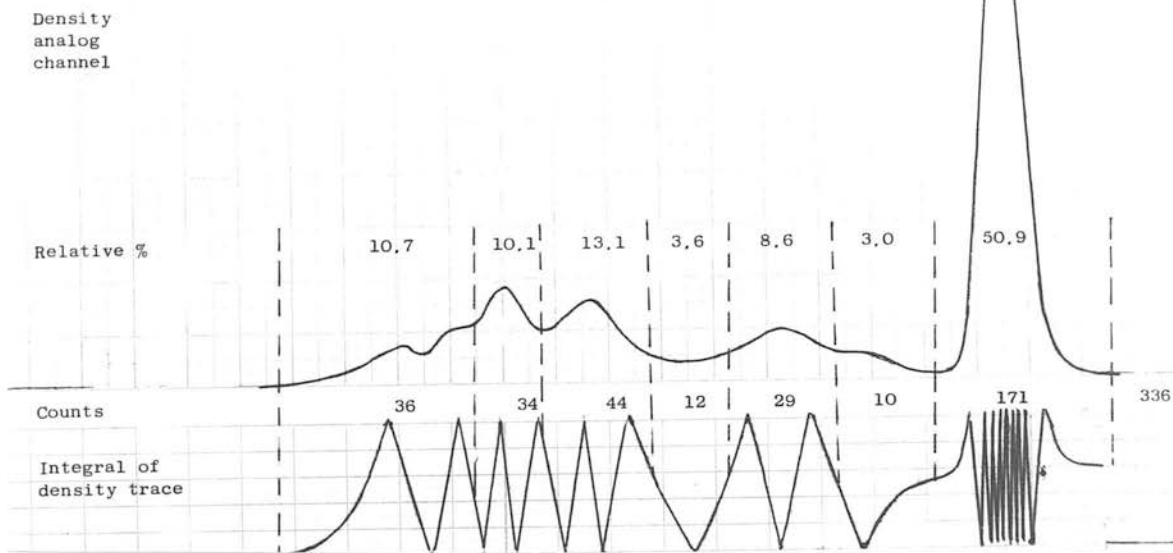


FIGURE 5.5

SERUM PROTEIN RECORDING AND ANNOTATION AFTER
AGAROSE GEL ELECTROPHORESIS.

FIGURE 5.6

Rf VALUES OF BOVINE SERUM PROTEINS AFTER
AGAROSE GEL ELECTROPHORESIS.

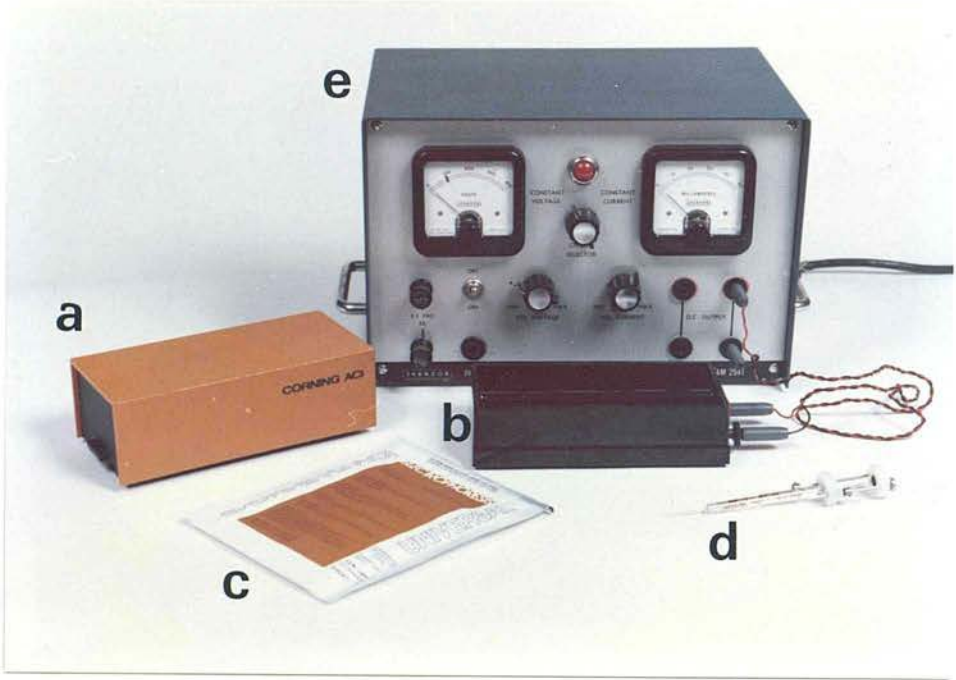
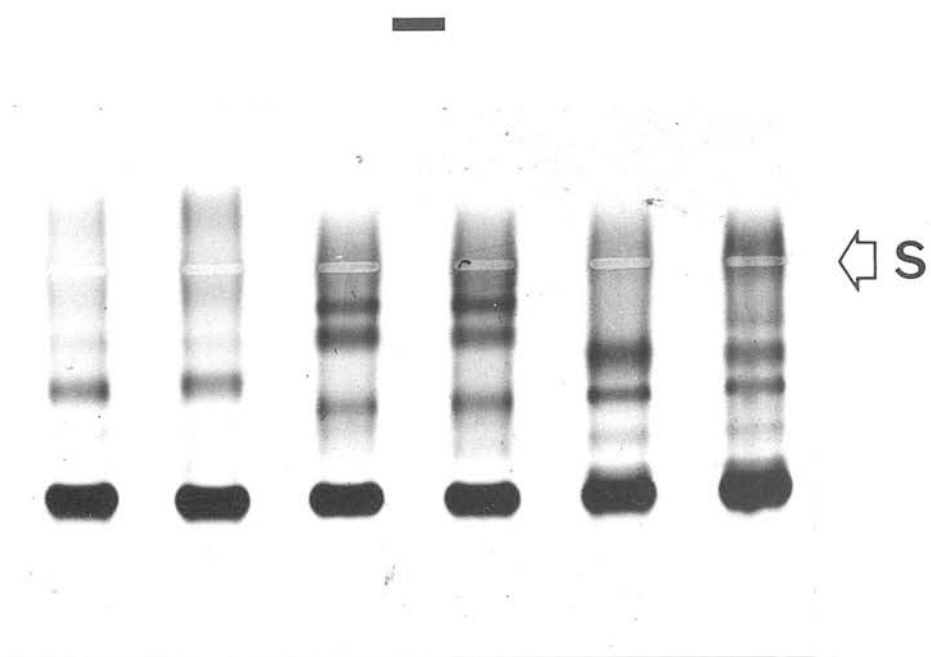


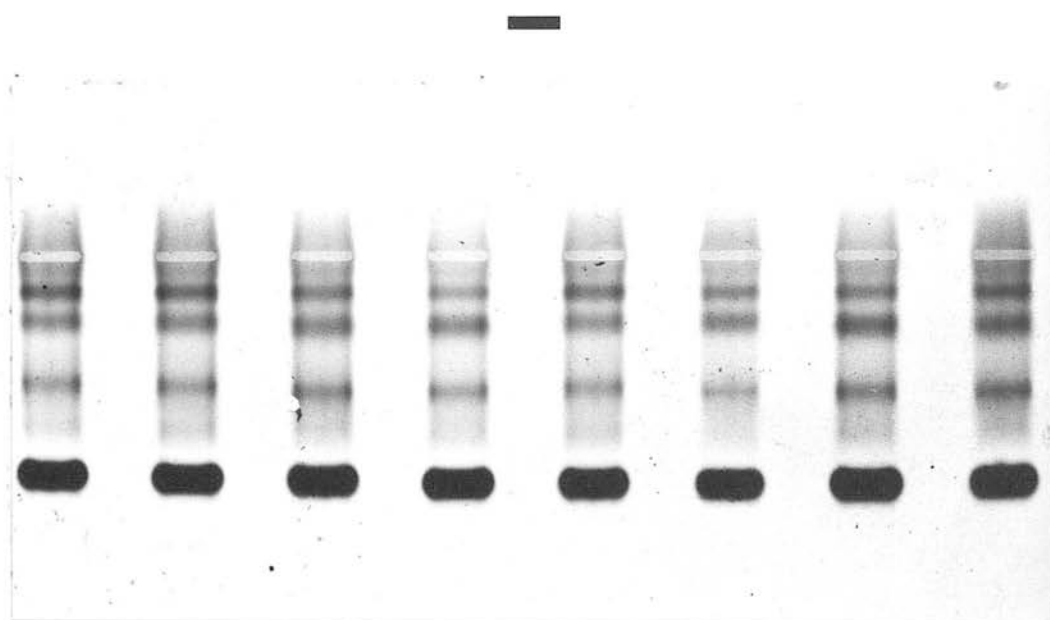
FIGURE 5.4

APPARATUS FOR AGAROSE GEL ELECTROPHORESIS

- (a) = cassette cover
- (b) = cassette base
- (c) = sealed pack of 2 agarose films
- (d) = microlitre sample dispenser with
disposable plastic tip
- (e) = power pack



+



+

FIGURE 5.2

AGAROSE GEL ELECTROPHORESIS OF SERUM

PROTEINS

Left to right

Ovine serum (2)

Bovine serum (2)

Equine serum (2)

FIGURE 5.3

AGAROSE GEL ELECTROPHORESIS OF SERUM

PROTEINS

Bovine serum (8)

CHAPTER VI

THE EFFECTS OF STORAGE ON PROTEIN ESTIMATIONSEXPERIMENT I

BIURET METHOD

Introduction

Since it was anticipated that in future experiments there may be delay between separation and analysis of serum for certain parameters, it was decided that any serum not analysed on the day of collection should be frozen at -15°C . For this reason the performances of the main methods used were checked against fresh serum and serum frozen for up to 7 days at -15°C to assess stability.

Materials and Methods

Initially 7 ml samples of venous blood from ten healthy adult Friesian cows were taken into plain Vacutainer[®] brand evacuated blood collection tubes (Becton-Dickinson U.K. Ltd., York House, Empire Way, Wembley, England) through 20 g x $1\frac{1}{2}$ " Vacutainer needles (Becton-Dickinson U.K. Ltd.) labelled, and allowed to clot at ambient temperature.

After approximately $2\frac{1}{2}$ hours the tubes were centrifuged at 1,300 g for 15 minutes and 3 x 1 ml aliquots of each serum were drawn off by pasteur pipette into 7 ml capacity screw-capped plastic bijou containers and appropriately identified with species, animal number and day number (i.e. day 0, day 3 and day 7). The day numbers 3 and 7 indicated the number of days each aliquot of serum was allowed to remain at -15°C before analysis.

The ten sera from day 0 (fresh serum) were analysed in duplicate for total protein content by the biuret method described

earlier (Materials and Methods V 1.(a) Page No 61). Results were expressed in g/l total protein. The same ten sera were analysed for total protein after the two storage periods of 3 days and 7 days at -15°C .

Results

TABLE 6.1a

TOTAL PROTEIN LEVELS - DIFFERENCE BETWEEN DUPLICATES.

				Paired 't' tests				
Observation	No.	\bar{x} g/l	s.	't'	p	df	$\bar{\Delta}$ g/l	Δ_s
1	10	70.9	2.83	0.260	0.8	9	0.06	0.72
2	10	70.8	3.10					

TABLE 6.1b

TOTAL PROTEIN LEVELS - EFFECTS OF STORAGE.

				Paired 't' tests				
Observation	No.	\bar{x} g/l	s	't'	p	df	$\bar{\Delta}$ g/l	Δ_s
A (Day 0)	10	70.9	2.83	0.801	>0.4	9	0.14	0.55
B (Day 3)	10	70.7	2.97					
A (Day 0)	10	70.9	2.83	0.632	>0.5	9	-0.24	1.19
C (Day 7)	10	71.1	2.77					

Statistical definitions

n = number of samples

\bar{x} = mean value

s. = standard deviation

p = level of significance

't' = 't' value

df = degrees of freedom

$\bar{\Delta}$ = mean difference between observations.

Δ_s = standard deviation of differences between observations.

In Table 6.1a the mean and standard deviation of duplicate observations of total protein values were calculated. A paired 't' test was carried out on the duplicate observations of each total protein value and the mean and standard deviation of the differences between observations were also calculated.

In Table 6.1b the mean and standard deviation for total protein in each group A, B and C was calculated and a paired 't' test was carried out on corresponding individual values between groups A and B and groups A and C. The mean and standard deviation of the differences between the same groups was also calculated. The probability of a significant difference in total protein level due to storage was thus determined.

Discussion

My results show the mean and total protein values from duplicate observations to be almost identical with the results of the paired 't' test yielding $p = 0.8$ (Table 6.1a).

From Table 6.1b the mean and standard deviation of values from groups A and B are shown to be very close and almost identical to those in Table 6.1a. The mean value from group C was very slightly higher than in groups A and B, but the results of the paired 't' test between A and C indicated that any differences were not statistically significant. The results of the paired 't' test between A and B also failed to demonstrate any significant difference in total protein values.

It was concluded from the statistical analysis of the results from this experiment that storage for up to 7 days at -15°C had no appreciable effect on total protein levels.

EXPERIMENT II

ALBUMIN BY B.C.G. DYE - BINDING METHOD

Materials and Methods

As in Experiment I aliquots of bovine serum were analysed on the day of collection and after storage at -15°C for 3 or 7 days. Albumin was determined by the B.C.G. dye-binding technique (See Chapt. V 1c Page 63), by reacting 25 μl of serum with 3.0 ml of working dye solution.

Absorbances were read at 628 nm at approximately 20 seconds and at 10 minutes after mixing serum with working dye reagent and were compared with those of a 30 g/l bovine albumin standard similarly treated. Results were expressed in g/l albumin.

ResultsTABLE 6.2a

SERUM ALBUMIN LEVELS - DIFFERENCE BETWEEN DUPLICATES, 'IMMEDIATE' AND 10 MINUTE B.C.G. REACTIONS.

Observation	No.	\bar{x} g/l	s.	Paired 't' tests				
				't'	p	df	$\bar{\Delta}$ g/l	Δs
'Immediate' Reaction (1)	10	36.4	2.26	0.902	>0.1	9	0.20	0.70
'Immediate' Reaction (2)	10	36.2	2.44					
10 Minute Reaction (1)	10	40.8	1.77	0.732	>0.4	9	0.17	0.73
10 Minute Reaction (2)	10	40.7	1.98					

TABLE 6.2b

SERUM ALBUMIN LEVELS - EFFECTS OF STORAGE ON 'IMMEDIATE' AND
10 MINUTE B.C.G. REACTIONS.

Observation	No.	\bar{x} g/l	s	Paired 't' tests				
				't'	p	df	$\bar{\Delta}$ g/l	Δ_s
A (Day 0) 'Immediate' Reaction	10	36.4	1.97	0.979	>0.3	9	-0.3	0.98
B (Day 3) 'Immediate' Reaction	10	36.5	1.85					
A (Day 0) 'Immediate' Reaction	10	36.4	1.97	0.771	>0.4	9	-0.14	1.46
C (Day 7) 'Immediate' Reaction	10	36.4	1.88					
A (Day 0) 10 Minute Reaction	10	40.8	1.77	0.305	>0.7	9	-0.13	1.16
B (Day 3) 10 Minute Reaction	10	40.9	1.67					
A (Day 0) 10 Minute Reaction	10	40.8	1.77	0.139	>0.8	9	-0.73	1.83
C (Day 7) 10 Minute Reaction	10	41.3	1.76					

For statistical definitions see page no. 73.

In Table 6.2a the mean and standard deviation for albumin values by the 'immediate' and 10 minute B.C.G. reactions were calculated. A paired 't' test was carried out on duplicate observations of each

albumin value by both the 'immediate' and 10 minute B.C.G. reactions. The mean and standard deviation of the differences between the duplicate observations was also calculated.

In Table 6.2b the mean and standard deviation of each group of results from A, B and C were calculated. A paired 't' test was carried out on corresponding albumin values between groups A and B and between groups A and C. The mean and standard deviation of the differences in albumin values between these groups was also calculated. The probability of a significant difference in albumin level occurring due to storage was thus calculated.

Discussion

Results calculated from absorbances read at approximately 20 seconds are attributed to the 'immediate' B.C.G. reaction first described by Gustafsson (1976) and later further investigated by Webster (1977). Results calculated from absorbances read at 10 minutes are attributed to the albumin reaction described by Doumas et al. (1971) who found the best agreement between albumin values by their dye binding method and by cellulose acetate electrophoresis after this period.

My results show (Table 6.2a) the means and standard deviations to be very close, between duplicate observations of albumin values by the 'immediate' reaction. A similar relationship was found between duplicate observations by the 10 minute B.C.G. reaction although the mean difference in albumin levels between the two reactions was approximately 4.4 g/l. In the next chapter (Chapter VII, Experiment IV) the differences in albumin levels in each of 3 species will be determined by the 'immediate' and 10 minute reactions to ascertain

whether there is any similarity with the figures determined in this experiment. In Table 6.2b the corresponding mean and standard deviations for albumin values by the 'immediate' and 10 minute B.C.G. reactions in groups A, B and C are shown to be very close. The results of paired 't' tests between corresponding individual albumin values from groups A and B and groups A and C obtained by both reactions failed to demonstrate any significant differences. It was concluded that storage for 7 days at -15°C had no appreciable effect on serum albumin levels by the B.C.G. method.

Although not statistically significant, the p values are slightly lower for 't' tests between groups A and B and A and C of the 'immediate' reaction, compared to p values for a similar comparison of the 10 minute reaction. This is probably due to the difficulty in manual standardisation of the 'immediate' (at approximately 20 seconds) reaction. At this point, the rate of change in absorbance of the serum/dye mixture is relatively fast, as the 'true albumin' reaction is almost instantaneous (Gustafsson, 1976) and after a few seconds the 'acute phase' reaction commences. This second reaction is virtually complete after 10-15 minutes, although Gustafsson recorded a further very small absorbance change up to 30 minutes. The 'acute phase' reactants were described as being non-albumin reacting proteins, probably mostly alpha globulin components such as orosomucoid and caeruloplasmin.

EXPERIMENT III

ALBUMIN BY AGAROSE GEL ELECTROPHORESIS

Introduction

As indicated in the introduction to Experiment I, it was anticipated that in experiments to follow, delays in the analysis of some parameters may be unavoidable and for this reason it is necessary to establish that storage at -15°C has no appreciable effect on results by the method in question. This is especially important with electrophoresis where direct absolute measurement is not possible and where relative percentage figures must be converted to absolute concentration. (e.g. total protein g/l x percentage albumin = albumin g/l).

Materials and Methods

Aliquots of bovine serum were analysed on the day of collection and after storage at -15°C for 3 days and 7 days. Albumin was determined by agarose gel electrophoresis. (See page No. 64)

Eight sera were electrophoresed in duplicate i.e. four pairs per plate (eight being the maximum number of samples able to be loaded on one plate). The same eight sera were electrophoresed after the storage periods of 3 days and 7 days at -15°C .

In addition, the eight sera were electrophoresed on two different plates on the same day to assess the interplate variation in the calculated relative percentage of albumin.

Results

TABLE 6.3a

SERUM ALBUMIN ELECTROPHORETIC PERCENTAGE - DIFFERENCE BETWEEN
DUPLICATES.

Observation	No.	\bar{x} %	s	Paired 't' tests				
				't'	p	df	$\bar{\Delta}$ %	Δs
1	8	49.8	2.20	0.283	>0.7	9	0.21	2.11
2	8	49.5	3.16					

TABLE 6.3b

SERUM ALBUMIN ELECTROPHORETIC PERCENTAGE - INTERPLATE VARIATION.

Observation	No.	\bar{x} %	s	Paired 't' tests				
				't'	p	df	$\bar{\Delta}$ %	Δs
Plate I	8	49.2	3.08	-1.577	>0.1	9	-0.48	0.851
Plate II	8	49.6	2.89					

TABLE 6.3c

SERUM ALBUMIN ELECTROPHORETIC PERCENTAGE - EFFECT OF STORAGE.

Observation	No.	\bar{x} %	s	Paired 't' tests				
				't'	p	df	$\bar{\Delta}$ %	Δs
A (Day 0)	8	49.7	2.54	0.943	>0.3	9	0.50	1.49
B (Day 3)	8	49.2	3.07					
A (Day 0)	8	49.7	2.54	0.359	>0.7	9	0.29	2.26
C (Day 7)	8	49.3	3.48					

For statistical definitions see page no. 73.

In Table 6.3a the mean and standard deviation were calculated on duplicate observations of each albumin relative percentage figure. A paired 't' test was carried out on duplicate observations of each albumin figure and the mean and standard deviation of the differences between duplicate observations were also calculated.

In Table 6.3b the mean and standard deviation of albumin percentage figures from two different plates I and II were calculated. A paired 't' test was carried out on corresponding individual albumin figures from each plate and the mean and standard deviation of the differences was also calculated.

In Table 6.3c the mean and standard deviation was calculated of albumin percentage figures from A, B and C. Paired 't' tests were carried out on corresponding individual albumin values from A and B and A and C. The mean and standard deviation of the differences between A and B and A and C were also calculated. The probability of a significant difference in albumin percentage due to storage was thus determined.

Discussion

The mean and standard deviation of duplicate observations on albumin percentage figures are shown to be very close (Table 6.3b). The results of a paired 't' test on these observations demonstrated no significant difference in albumin percentage figures.

Table 6.3b shows the mean and standard deviation of albumin percentage figures from two different plates to be almost identical and a paired 't' test between corresponding individual albumin percentage figures indicated no significant difference in figures

between Plate I and Plate II.

Table 6.3c shows the mean and standard deviation of albumin percentage figures in A, B and C to be very close and paired 't' tests between individual albumin percentage figures from A and B and A and C demonstrated that any differences were not significant.

It was concluded from the statistical analysis of the results from this experiment that storage for up to 7 days at -15°C had no appreciable effect on the albumin relative percentage figure calculated after agarose gel electrophoresis.

Provided that aliquots of fresh serum are stored at -15°C , the figures from the experiments in this chapter show that storage for up to 7 days has no appreciable effect on total protein, albumin or electrophoretic results and therefore if necessary, this storage procedure may be safely adopted with serum for future experiments.

CHAPTER VII

EXPERIMENT IV

COMPARISON OF BOVINE, OVINE AND EQUINE SERUM ALBUMIN LEVELS
BY THE 'IMMEDIATE' AND 10 MINUTE B.C.G. REACTIONS.

Introduction

In a previous experiment (Experiment II, Chapter VI) it was noted that when bovine serum albumin was determined by both the 'immediate' and 10 minute B.C.G. reactions, there was a considerable difference between the two sets of results, with the mean albumin value by the latter reaction being 4.0 g/l (approximately 10%) higher than the mean value by the 'immediate' reaction. The following experiment was set up to determine the differences in albumin levels using the two B.C.G. reactions. Serum from three different species was used to ascertain whether the magnitude of the differences was similar to those found in Experiment II.

Materials and Methods

Blood samples were obtained from normal animals from each of three species, bovine, ovine and equine, in a similar manner to that described in Chapter VI, Page 72 . The breeds and age groups of the animals were as follows:-

Bovine - Blue-grey cows, 2-5y.o. (19)

Ovine - Greyface gimmers, 2 y.o. (16)

Equine - Eriskay ponies, 2-6 y.o. (19)

Serum from each species was analysed for albumin by the B.C.G. dye-binding method using the two different reaction times. Species specific standards were employed for comparison. (See Page No. 63)

Statistical analysis of the albumin values in each of the three

species, using the two different reaction times, were carried out using the Student's paired 't' test procedure.

Results

TABLE 7.1a

SERUM ALBUMIN BY THE B.C.G. METHOD - 'IMMEDIATE' AND 10 MINUTE REACTIONS COMPARED.

Species	No	Method	\bar{x} g/l	s	Paired 't' tests					Range g/l
					't'	p	df	$\bar{\Delta}$	Δs	
Bovine	19	A	30.4	2.53	-16.612	<0.001	18	-4.26	1.11	25.4-34.2
	19	B	34.4	2.16						

TABLE 7.1b

Ovine	16	A	29.5	3.40	-8.116	<0.001	15	-2.16	1.06	19.2-33.7
	16	B	32.1	3.07						

TABLE 7.1c

Equine	19	A	22.2	2.49	-16.20	<0.001	18	-2.33	0.63	17.5-26.5
	19	B	24.3	2.22						

For statistical definitions see page no. 73.

Method key - A 'Immediate' B.C.G. Reaction.

B 10 Minute B.C.G. Reaction.

In Tables 7.1a, b and c the mean and standard deviation of albumin values obtained using the two different B.C.G. reaction times were calculated for each species. Paired 't' tests were also carried out between individual values by the two reactions. The statistical significance of the differences in albumin results by the two reactions was calculated for each species.

Discussion

The results from Table 7.1a show a similar pattern to those from Table 6.2a, Chapter VI, in that the mean of the differences of bovine serum albumin by the 10 minute B.C.G. reaction was 4.3 g/l higher than by the 'immediate' B.C.G. reaction. The results of a paired 't' test indicated that these differences were highly significant ($p = < 0.001$).

From a similar comparison, the mean of the differences with ovine and equine serum albumin, (Tables 7.1b and 7.1c) although statistically highly significant, were found to be less in absolute terms than those with bovine serum albumin. With ovine serum, the 10 minute B.C.G. reaction gave values 2.2 g/l higher than the 'immediate' B.C.G. reaction and with equine serum, 2.3 g/l higher than the 'immediate' B.C.G. reaction.

It is clear from the results of this comparison that for the species studied, there are statistically significant differences in serum albumin results between the 'immediate' and 10 minute B.C.G. reactions. It is proposed in later experiments to ascertain which of the two reactions will provide results which are closest to those by a reference technique ('rocket' immunoelectrophoresis)

for serum albumin.

It is also proposed in later experiments outside this work, to investigate the possible reasons for the mean differences in albumin levels by the two B.C.G. reaction times with bovine serum, being almost twice those of ovine and equine serum.

CHAPTER VIII

EXPERIMENT VMODIFICATION OF THE 'IMMEDIATE' B.C.G. DYE-BINDING REACTION
TO A ONE STEP PROCEDURE.Introduction

Since in future experiments the albumin values as obtained by the 'immediate' and 10 minute B.C.G. reactions are to be compared to those by agarose gel and 'rocket' immunoelectrophoresis, it was desirable to find a simple, reproducible method of directly obtaining the 'immediate' reaction absorbance readings. It has been stated that the 'immediate' B.C.G. reaction with serum is the more specific for albumin while higher values by the slower reaction of approximately 10 minutes duration is attributed to the reaction of other globulins with the dye causing a further increase in absorbance.

To keep the time interval between mixing dye and serum to a minimum it was decided to eliminate one step of the procedure i.e. the transfer of dye/serum mixture from reaction tube to cuvette prior to taking an absorbance reading. It was decided to retain the same serum/dye ratio i.e. 1:200 but reduce the actual volumes proportionately so that the reaction could be performed directly in the measurement cuvette. (See Fig. 8.1). This would constitute not only a saving in glassware and reagents but would ensure that timing of absorbance readings especially the 'immediate' (20-30 seconds) would be easier to standardise. Similarly timed absorbance readings of a bovine albumin standard were taken for comparison. Doumas, Watson and Biggs (1971) found the full colour development of aqueous albumin solutions to be instantaneous, but Gustafsson (1976) noted

FIGURE 8.1

PERSPEX CUVETTE RACK

A = base set at 3.5 mm depth.

Positions 1-7 in rack - cuvettes containing 3ml B.C.G. working dye solution. Positions 8 and 9 illustrate change in colour of working dye solution when serum B and low albumin standard are added.

Photograph also shows 1-20 μ l adjustable sampler with disposable plastic tip.



that it took up to 1 minute for a final absorbance value to be reached.

The following experiment was set up to ascertain if there would be any significant difference in serum albumin results if the original procedure as detailed in Materials and Methods Page No. 63, was substituted with this modification.

Materials and Methods

Eight samples of bovine serum were obtained in a similar manner to that described in Chapter VI, Experiment I. Each serum sample was dispensed in 2 x 1 ml aliquots into 7 ml size plastic bijou containers and labelled with the appropriate animal number. One serum sample from each pair was further labelled with procedure '1' while the other was marked with procedure '2'.

A cuvette rack designed to hold up to 20 x 1 cm plastic spectrophotometer cuvettes was constructed in 2.5 mm thickness perspex with overall dimensions 23.5 cm x 7.5 cm x 5.5 cm. The base A was set at 3.6 mm depth and two rows of 10 x 18 mm diameter holes were drilled in the top section so that when seated the cuvettes would protrude by 1 cm to allow easy removal. (See Fig. 8.1) A 23.5 cm x 1.5 cm strip of perspex was attached to the under surface of the top section in such a way that the edges of the strip overlapped part of each row of holes. This arrangement ensured that the cuvettes remained upright and steady when placed in the rack.

The working B.C.G. dye solution used was similar to that described in Materials and Methods Page No. 63.

Procedure '1'. The eight samples of bovine serum were analysed by the method described in Materials and Methods Page No. 63.

with the albumin concentration of each being calculated against at 31.8 g/l bovine albumin standard analysed in the same way.

'Immediate' (< 30 seconds) and 10 minutes absorbance readings were taken at 628 nm, the reaction taking place in a glass test tube prior to transfer to a plastic cuvette for absorbance measurement.

Procedure '2'. The same eight samples of bovine serum were analysed in a similar manner to procedure 'B' except that the reaction was performed directly in the measurement cuvette and 3.0 ml of working dye solution were reacted with 15 μ l of serum instead of 5 ml and 25 μ l respectively in Procedure '1'.

Results

TABLE 8.1

SERUM ALBUMIN BY B.C.G. METHOD - PROCEDURES '1' AND '2' COMPARED.

Observation	Method	No.	\bar{x} g/l	s	't'	p	df	$\bar{\Delta}$ g/l	Δs
Procedure '1'	'Immediate' Reaction	8	32.9	1.40	0.961	>0.3	7	0.41	1.21
Procedure '2'	'Immediate' Reaction	8	32.5	1.14					
Procedure '1'	10 Minute Reaction	8	36.2	1.51	0.935	>0.3	7	0.40	1.20
Procedure '2'	10 Minute Reaction	8	35.8	1.14					

For statistical definitions see page 73.

In Table 8.1, the mean and standard deviation of albumin values were calculated, applying Procedure '1' and Procedure '2' to the 'immediate' and 10 minute B.C.G. reactions. Paired 't' tests were carried out between corresponding individual values using each of the two reaction times and procedures. By applying Procedures '1'

and '2' to the 'immediate' reaction and also to the 10 minute the statistical significance in albumin results was calculated.

Discussion

The mean albumin values between procedures '1' and '2' using the 'immediate' and 10 minute reactions remain very close.

A paired 't' test between corresponding albumin values by procedures '1' and '2' for both reactions indicated that there was no significant difference in results between the procedures. It was therefore considered that the modified procedure would be employed in future when serum albumin is to be determined by the B.C.G. dye-binding method.

This modification to the original procedure offers clear advantages when the 'immediate' B.C.G. reaction is to be observed. Since the reaction progresses very quickly in the initial stage i.e. immediately after mixing serum and dye, if an absorbance reading is to be taken quickly a one-step procedure is preferable. Although it is just possible to perform the reaction in a test tube and complete the transfer of contents to a measurement cuvette in approximately 15 seconds it allows little leeway and requires considerable dexterity. Other advantages in using the modified procedure are savings in glassware and reagents which could be considerable if large numbers of samples are involved.

It was noted that colour development of the aqueous albumin standard was virtually complete at approximately 30 seconds. A further very slight increase in absorbance was recorded up to approximately 1 minute but would probably not have been detectable using a less sensitive spectrophotometer.

CHAPTER IX

EXPERIMENT VI.1

DETERMINATION OF SERUM ALBUMIN BY 'ROCKET' IMMUNOELECTROPHORESIS

Introduction

In future experiments albumin levels obtained by the bromocresol green dye-binding method will be compared to those by agarose gel electrophoresis. Although recent technical advances have to a certain extent allowed workers to minimise interferences and phenomena as discussed in the Review of the Literature, neither of the two methods mentioned could be considered truly specific for albumin. For this reason a more specific method for the determination of serum albumin was employed to serve as a reference for the other two. 'Rocket' immunoelectrophoresis is widely recognised as being such a method.

An investigation is described here to determine suitable conditions for the 'rocket' immunoelectrophoresis method to allow future comparisons to be drawn with this method, dye-binding and electrophoresis for the determination of serum albumin.

Certain conditions as described by Weeke (1973) were followed, such as applying a high voltage of 280 volts over a 2-4 hour period to produce 'fast rockets'. The object was to determine the overall conditions needed to ensure reproducible results in the shortest possible time.

Experiment

The method described earlier (page no. 67) was followed for the preparation of plates. Anti-bovine albumin was used to prepare the 1% gel containing antibodies.

Dilutions of $1/300$ and $1/200$ of bovine albumin standards were prepared in electrophoresis buffer. The actual concentrations of the bovine albumin standards had been determined by the biuret method described earlier (page no. 61). Two plates were run with dilutions of bovine albumin standards to cover a range of approximately 10-40 g/l, one for $3\frac{1}{2}$ hours and one for $2\frac{1}{2}$ hours at 280 volts.

Results

TABLE 9.1

DETERMINATION OF 'ROCKET' PEAK HEIGHTS OF ALBUMIN STANDARDS AFTER
'ROCKET' IMMUNOELECTROPHORESIS (I.E.P.)

Actual Concentration of Bovine Albumin Standard g/l	Dilution of Standard	(a) 'Rocket' I.E.P. Peak Height mm	(b) 'Rocket' I.E.P. Peak Height mm
12.5	$1/300$	6.5	10.0
	$1/200$	10.0	16.0
21.8	$1/300$	13.5	22.0
	$1/200$	20.0	32.0
31.0	$1/300$	20.0	32.0
	$1/200$	28.5	45.0
39.5	$1/300$	25.5	40.0
	$1/200$	36.5	57.0

(a) 280 volts for $2\frac{1}{2}$ hours.

(b) 280 volts for $3\frac{1}{2}$ hours.

From the results obtained a standard curve was constructed (see

FIGURE 9.1

'ROCKET' IMMUNOELECTROPHORESIS

ALBUMIN STANDARD CURVE

280 volts $2\frac{1}{2}$ hours

Buffer ionic strength 0.02

Dilution of albumin standards $1/200$

ROCKET
IMMUNOELECTROPHORESIS
ALBUMIN STANDARD
CURVE.

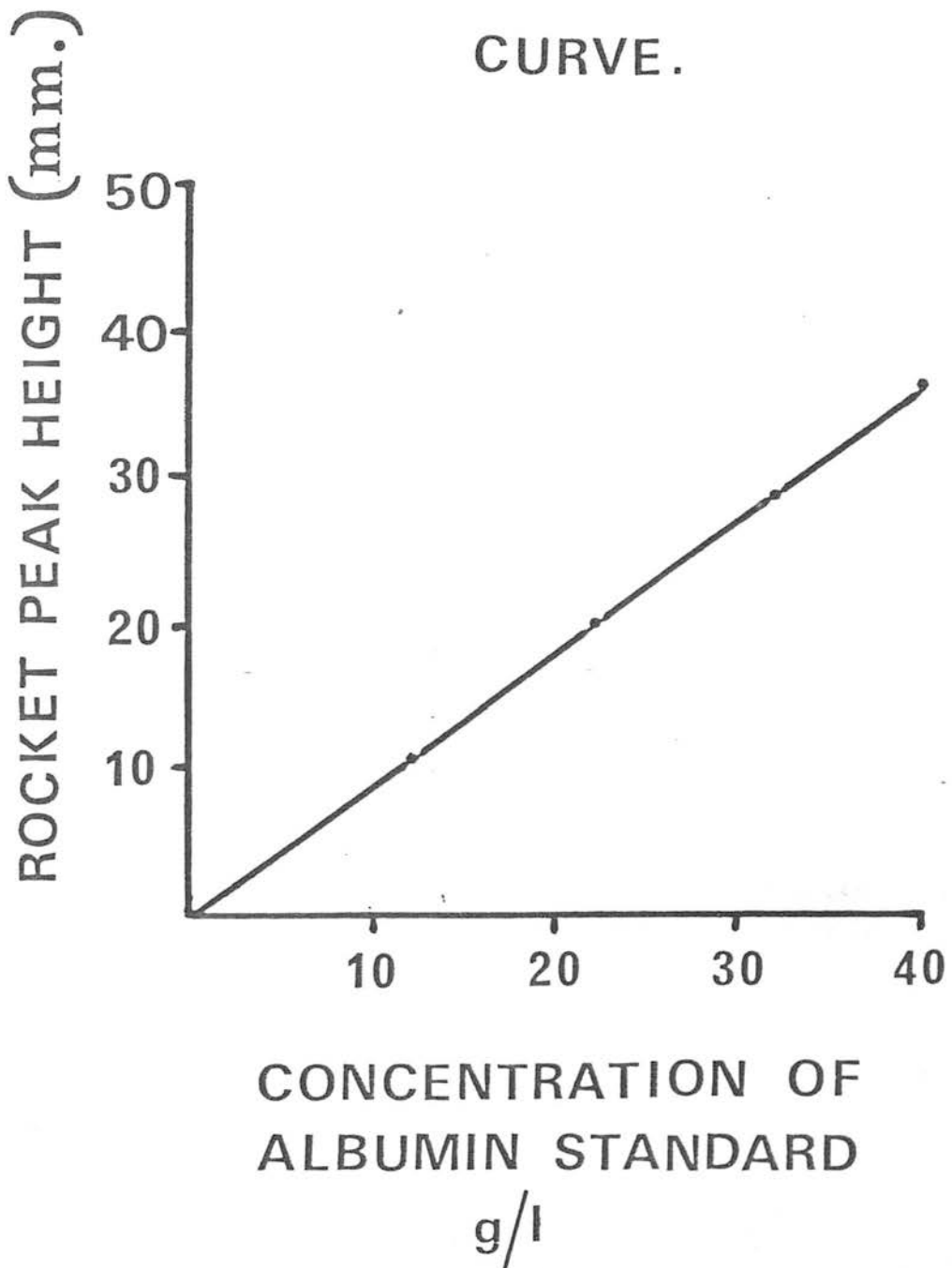


Fig. 9.1). The figures from column (a) of Table 9.1 were used, with the peak height of each $1/200$ dilution of albumin standard plotted against its actual concentration.

Discussion

The $1/200$ dilutions of albumin standards under the conditions described in Table 9.1 column (a) provided good 'rocket' shaped precipitates with a suitable peak height, that is the height in mm was close to the actual concentration of each albumin standard e.g. 21.8 g/l albumin standard had peak height of 20 mm. It was decided to adopt these conditions in later experiments.

Fig. 9.1 demonstrates that there was a linear relationship between 'rocket' peak height and albumin concentration up to at least 40 g/l when the figures for $1/200$ dilutions of standards in Table 9.1 column (a) were applied.

EXPERIMENT VI.2REPRODUCIBILITY AND INTERPLATE VARIATION OF SERUM ALBUMIN VALUES
BY 'ROCKET' IMMUNOELECTROPHORESIS.Introduction

Eight samples of fresh bovine serum were subjected to 'rocket' immunoelectrophoresis and were distributed on four plates A,B,C and D in such a way as to allow assessment of the reproducibility and interplate variation of serum albumin values obtained.

Experiment

8 x 5 μ l replicates of a $1/200$ dilution of a single sample of bovine serum were applied to sample wells on Plate A together with similar volumes of bovine albumin standards in duplicate. The concentrations of the standards were 21.8 g/l and 31.0 g/l.

4 sets of 5 μ l duplicate samples of $1/200$ dilutions of four different bovine sera were applied to sample wells on Plate B together with similar bovine albumin standards as on Plate A.

Another plate (Plate C) was loaded with four bovine sera in a similar manner to Plate B and with a similar distribution of bovine albumin standards.

Plate D was loaded with 8 x 5 μ l samples of $1/200$ dilutions of the eight different bovine sera contained on Plates B and C, with a similar distribution of bovine albumin standards. 'Rocket' immunoelectrophoresis was carried out under the conditions described in Experiment VI.1 and serum albumin results on each plate were calculated against the mean peak height of the 31.5 g/l bovine albumin standard as follows:-

$$\text{serum albumin conc.} = \frac{\text{peak height (mm)}}{\text{peak height of standard (mm)}} \times \text{conc. of standard (g/l)}$$

ResultsTABLE 9.2

'ROCKET' IMMUNOELECTROPHORESIS - REPRODUCIBILITY AND INTERPLATE
VARIATION OF ALBUMIN VALUES.

REPLICATES

Plate	Serum No	No	\bar{x} g/l	s	Paired 't' tests					Range g/l
					't'	p	df	$\bar{\Delta}$ g/l	Δs	
A	1	8	29.9	0.62	-	-	-	-	-	-

TABLE 9.3

DUPLICATES

B	Observation 1 1-4	4	29.7	1.66	0.293	>0.7	3	0.12	0.85	28.5 - 32.0
B	Observation 2 1-4	4	29.6	1.31						

TABLE 9.4

DUPLICATES

C	Observation 1 5-8	4	27.6	1.65	0	>0.9	3	0	0.91	26.5 - 30.0
C	Observation 2 5-8	4	27.6	1.38						

For statistical definitions see page no. 73.

TABLE 9.5

INTERPLATE VARIATION

Plate	Serum No	No	\bar{x} g/l	s	Paired 't' tests					Range g/l
					't'	p	df	$\bar{\Delta}$ g/l	Δs	
B	Observation 1 1-4	4	29.7	1.66	0.293	>0.8	3	0.12	0.85	28.5 - 30.0
D	Observation 3 1-4	4	29.6	1.31						
B	Observation 2 1-4	4	29.6	1.31	0	>0.9	3	0	0.71	28.5 - 30.0
D	Observation 3 1-4	4	29.6	1.31						

TABLE 9.6

INTERPLATE VARIATION

C	Observation 1 5-8	4	27.6	1.65	0	>0.9	3	0	0.91	26.5 - 30.0
D	Observation 2 5-8	4	27.6	1.38						
C	Observation 2 5-8	4	27.6	1.38	0.926	>0.4	3	0.5	1.08	26.5 - 30.0
D	Observation 3 5-8	4	27.1	0.63						

For statistical definitions see page no. 73.

In Table 9.2 the mean and standard deviation of the eight

replicate bovine serum albumin values were calculated.

In Tables 9.3 and 9.4 the duplicate observations of albumin values from sera 1-4 and 5-8 respectively were formulated to provide a mean and standard deviation for each set of observations. A paired 't' test was also carried out between each set of duplicate observations and the mean and standard deviation of the differences was also calculated.

In Tables 9.5 and 9.6 the observations utilised in Tables 9.3 and 9.4 were combined with a further set of single observations on sera 1-8 to provide three sets of data for sera 1-4 and also for sera 5-8. Two of the three sets of data are duplicated on plates B and C, whilst the third set is provided by the results from Plate D. The mean and standard deviation from the three observations in Tables 9.5 and 9.6 were calculated. A paired 't' test was carried out on corresponding individual values from Observation 1 and Observation 2 and Observation 1 and Observation 3 in each case. The mean and standard deviation of the differences between these observations was also calculated.

Discussion

The results from Table 9.2 represent a high degree of reproducibility for the technique. The eight replicate albumin values had a standard deviation of 0.62 which gives a coefficient of variation ($\frac{100 \times s}{\bar{x}}$) of 2.07%.

In Tables 9.3 and 9.4 the mean albumin values and standard deviations from each set of duplicate observations were almost identical and no statistically significant differences were found between duplicates.

In Tables 9.5 and 9.6 the mean albumin values and standard deviations from each set of observations on the same sera were also very similar. No statistically significant differences in albumin levels were found when each group of sera were analysed on two different plates.

Statistical analysis of the results from this experiment illustrate that using the experimental conditions stated, a high degree of reproducibility can be achieved with this technique.

CHAPTER X

EXPERIMENT VII

BOVINE, OVINE AND EQUINE SERUM ALBUMIN VALUES. 'ROCKET'
IMMUNOELECTROPHORESIS AND THE BROMOCRESOL GREEN DYE-BINDING
METHOD COMPARED.

Introduction

Since the main objective of this work is to draw a comparison between albumin values obtained from various species by a bromocresol green dye-binding method and by agarose gel electrophoresis it is necessary to establish that at least one of the two methods is in good agreement with a reference technique for the measurement of serum albumin. As discussed earlier the reference technique employed in this case is 'rocket' immunoelectrophoresis. Although this technique is more specific for albumin determination than dye-binding or conventional electrophoretic methods, it suffers from the disadvantages over the other two of increased time, cost and complexity.

As stated in the Review of the Literature other workers in recent years have drawn favourable comparisons between results obtained for human serum albumin concentration by 'rocket' immunoelectrophoresis and the 'immediate' bromocresol green reaction. Good comparisons have also been achieved between the latter results and those by electrophoretic methods.

It is proposed here to determine whether a similar favourable comparison can be drawn when animal sera are used.

Materials and Methods

Ten samples of serum from normal animals from each of the three species were obtained, in a similar manner to that described

in Chapter VI, and dispensed into appropriately labelled 7 ml capacity bijou containers. The breeds and age groups of the species bled were:-

Bovine - 2 y.o. Blue grey heifers.

Ovine - 2 y.o. Grey face gimmers.

Equine - 2-4 y.o. Thoroughbred and T.B. x horses.

The ten serum samples from each species were analysed for albumin by 'rocket' immunoelectrophoresis and by the bromocresol green dye-binding method as described on pages and respectively. Albumin values by the 'immediate' and 10 minute B.C.G. reactions were compared with those by 'rocket' immunoelectrophoresis. Species specific standards were used in both methods to calculate the albumin concentrations.

Results

TABLE 10.1

SERUM ALBUMIN LEVELS IN DIFFERENT SPECIES - 'ROCKET'

IMMUNOELECTROPHORESIS AND B.C.G. DYE-BINDING TECHNIQUES COMPARED.

Species	No	Method	\bar{x} g/l	s	Paired 't' tests					Range g/l
					't'	p	df	$\bar{\Delta}$ g/l	Δs	
Bovine	10	A	28.4	1.81	1.358	>0.2	9	-0.44	1.02	26.0-30.7
	10	B	28.9	1.48						25.8-31.0
	10	A	28.4	1.81	-10.61	<0.001	9	-4.22	1.26	26.0-30.7
	10	C	32.7	1.06						31.2-34.2

For statistical definitions see page no. 73.

Method key - A 'Rocket' Immunoelectrophoresis.

B 'Immediate' B.C.G. Reaction.

C 10 Minute B.C.G. Reaction.

TABLE 10.2

SERUM ALBUMIN LEVELS IN DIFFERENT SPECIES - 'ROCKET'

IMMUNOELECTROPHORESIS AND B.C.G. DYE-BINDING TECHNIQUES COMPARED.

Species	No	Method	\bar{x} g/l	s	Paired 't' tests					Range g/l
					't'	p	df	$\bar{\Delta}$ g/l	Δs	
Equine	10	A	28.8	1.24	0.542	>0.6	9	0.19	1.11	26.6-31.0
	10	B	29.0	1.79						27.0-31.9
	10	A	28.8	1.24	-8.07	<0.001	9	-2.51	0.98	26.6-31.0
	10	C	31.4	1.71						28.6-32.6

TABLE 10.3

Ovine	10	A	29.8	3.73	0.817	>0.4	9	0.42	1.62	20.0-32.0
	10	B	29.4	4.16						19.2-33.7
	10	A	29.8	3.73	-6.08	<0.001	9	-2.71	1.41	20.0-32.0
	10	C	32.5	3.59						23.7-37.1

For statistical definitions see page no. 73.

Method key - A 'Rocket' Immunoelectrophoresis.

B 'Immediate' B.C.G. Reaction.

C 10 Minute B.C.G. Reaction.

In Tables 10.1, 10.2 and 10.3 the mean and standard deviation of albumin levels by 'rocket' I.E.P. and 'immediate' and 10 minute B.C.G. reactions were calculated for each of the three species. Paired 't' tests were also carried out on individual albumin values obtained by the 'rocket' and each of the two B.C.G. techniques.

The mean and standard deviation of the differences in each of the comparisons was also calculated.

Discussion

The figures show that the mean serum albumin values for each species studied, by 'rocket' immunoelectrophoresis and by the 'immediate' B.C.G. reaction bear a close similarity. Paired 't' tests between corresponding individual albumin values by these two procedures showed no significant differences but paired 't' tests between individual albumin values for each species by 'rocket' immunoelectrophoresis and by the B.C.G. 10 minute reaction did show significant differences.

My figures show that serum albumin results for the three species obtained by the 'immediate' B.C.G. procedure are in good agreement with the reference technique i.e. 'rocket' immunoelectrophoresis.

However comparison of results by 'rocket' immunoelectrophoresis and the 10 minute B.C.G. method showed statistically significant differences, presumably due to non specific dye binding. It has also been recorded that alpha and beta-globulin fractions were found to react with working B.C.G. solution, producing significant 'albumin' peaks using a continuous automated method (Webster, 1974). Webster noted that alpha globulin only produced about one-fifth and beta globulin only one-tenth of the colour intensity with B.C.G. reagent when compared to that produced by an equivalent amount of albumin.

It is proposed in a later experiment to ascertain whether certain globulin fractions from the three animal species studied will react with the working B.C.G. dye reagent to produce any increase in absorbance.

CHAPTER XI

EXPERIMENT VIIICOMPARISON BETWEEN BOVINE, OVINE AND EQUINE ELECTROPHORETICSERUM ALBUMIN VALUES AND THOSE BY THE 'IMMEDIATE' AND 10 MINUTEB.C.G. REACTIONS.

USING SPECIES SPECIFIC STANDARD B.C.G. CURVES.

Introduction

It is clear from the results of preceding experiments that serum albumin can be determined rapidly, reproducibly and accurately in normal serum when the 'immediate' B.C.G. reaction is employed. However, for the occasions on which it is not necessary for albumin to be estimated as a part of a complete serum protein profile, it was considered useful to ascertain whether there would be an agreement in absolute terms between the electrophoretic albumin values and those by the B.C.G. dye-binding method.

In this experiment the serum albumin levels in some normal animals from three species are compared using agarose gel electrophoresis and the 'immediate' and 10 minute B.C.G. reactions.

Materials and Methods

The same numbers and groups of sera which were used in Experiment IV were employed in this experiment.

Each serum was analysed for albumin by agarose gel electrophoresis (as described on page 64) and by the B.C.G. dye-binding method (as described on page 88) using species specific albumin standards. Total protein was determined by the biuret reaction (as described on page 61).

As stated previously in Chapter V (page 65), up to eight

samples of serum can be electrophoresed simultaneously on each agarose film, but since the numbers of bovine and equine sera were not in exact multiples of eight, two films were used in each case to accommodate the first two groups of eight sera from these species whilst a third film accommodated the remaining three sera from each.

The electrophoretic albumin values were calculated in absolute terms by relating each albumin percentage value (as described in Chapter VI page 79) to its total protein level expressed in g/l.

Statistical analysis of the albumin values obtained by the two methods was carried out using the Student's 't' test procedure. Linear regression analysis was carried out on the results obtained by electrophoresis and the 'immediate' B.C.G. reaction.

ResultsTABLE 11.1

SERUM ALBUMIN - AGAROSE GEL ELECTROPHORESIS AND THE 'IMMEDIATE'
AND 10 MINUTE B.C.G. REACTIONS COMPARED.

Species	No	Method	\bar{x} g/l	s	Paired 't' tests					Range g/l
					't'	p	df	$\bar{\Delta}$ g/l	Δs	
Bovine	19	1	30.8	2.74	1.230	>0.2	18	0.52	1.83	26.3-35.7
	19	2	30.4	2.53						25.4-34.2
	19	1	30.8	2.74	-7.260	<0.001	18	-3.74	2.24	26.3-35.7
	19	3	34.4	2.16						30.8-38.0

TABLE 11.2

Ovine	16	1	29.8	3.31	1.039	>0.3	15	0.33	1.29	21.1-35.5
	16	2	29.5	3.40						19.2-34.5
	16	1	29.8	3.31	-6.510	<0.001	15	-1.80	1.11	21.1-25.5
	16	3	32.1	3.07						23.7-37.1

TABLE 11.3

Equine	19	1	22.7	2.53	1.177	>0.2	18	0.46	1.71	18.9-28.1
	19	2	22.2	2.49						17.5-26.5
	19	1	22.7	2.53	-3.999	<0.001	18	-1.75	1.91	18.9-28.1
	19	3	24.3	2.22						20.1-28.0

For statistical definitions see page 73.

Method key - 1 Electrophoresis.

2 'Immediate' B.C.G. Reaction.

3 10 Minute B.C.G. Reaction.

TABLE 11.4

SERUM ALBUMIN - REGRESSION EQUATIONS FOR ELECTROPHORESIS AND THE
'IMMEDIATE' B.C.G. REACTION COMPARED FOR THREE SPECIES.

$$\underline{y \text{ (B.C.G.)} = m \times (\text{electrophoresis}) + c}$$

<u>Species</u>	<u>No.</u>	<u>Regression Equations</u>
Bovine	19	$y = 0.70 x + 8.75 \text{ (} r = 0.760 \text{)}$
Ovine	16	$y = 0.95 x + 1.08 \text{ (} r = 0.928 \text{)}$
Equine	19	$y = 0.76 x + 5.08 \text{ (} r = 0.764 \text{)}$

Statistical definitions

m = regression coefficient (slope).

c = intercept of regression line on x axis.

r = correlation coefficient.

Tables 11.1, 2 & 3 show the mean and standard deviation for albumin values in each of the three species, by electrophoresis, 'immediate' and 10 minute B.C.G. reactions. The results of paired 't' tests between electrophoretic albumin values and the two different B.C.G. reaction times are also included. In bovine, equine and ovine serum, the statistical differences in albumin levels between electrophoresis and the 'immediate' B.C.G. reaction were $p = >0.2$, $p = >0.3$ and $p = >0.2$ for bovine, ovine and equine serum respectively. The differences in albumin levels between electrophoresis and the 10 minute B.C.G. reaction were $p = <0.001$, $p = <0.001$ and $p = <0.001$ for bovine, ovine and equine serum respectively. In the former comparison the mean differences were, in absolute terms 0.52 g/l, 0.33 g/l and 0.46 g/l respectively and in the latter comparison, -3.44 g/l, -1.80 g/l and -1.75 g/l.

The regression equations for albumin by the 'immediate' B.C.G. reaction (y) and by electrophoresis (x) were formulated and are included in Table 11.4.

EXPERIMENT IX

USING SPECIES SPECIFIC AND NON-SPECIFIC (BOVINE) ALBUMIN STANDARD B.C.G. CURVES.

Introduction

It has been shown that the absorbance/concentration standard curves for the three species of albumin Fr.V which were studied by the B.C.G. dye binding method, were not identical (see Chapter V, Fig.5.1). Equivalent amounts of pure albumin from each of the three species reacted differently, with equine serum binding with the dye to produce the greatest unit absorbances, while ovine albumin produced the least in terms of unit absorbance.

This experiment was undertaken to determine the differences between electrophoretic serum albumin values and those by the 'immediate' B.C.G. reaction by reading serum/dye absorbance values from equine and ovine species against their specific albumin standard curves and also against the bovine albumin standard curve. (The comparison between electrophoretic albumin values and 'immediate' B.C.G. albumin values has already been investigated in the previous experiment, but these figures will also be included with the results of this experiment, for comparative purposes.

From their results, Gentry and Lumsden (1978) found that provided the 'immediate' reaction was measured, the B.C.G. method was in good agreement with their agarose gel electrophoretic method, irrespective of whether bovine albumin or species specific

albumin was used to compute the albumin concentration. They suggested that although differences in absolute values may be obtained between the two methods, these were consistent over a wide range and it would therefore probably be satisfactory for most routine laboratories to use only a bovine standard albumin curve, which would offer subsequent savings in cost and reagent preparation time.

Materials and Methods

The same methods and sera which were used in the previous experiment were employed in this experiment. The electrophoretic albumin values and the 'immediate' reaction B.C.G. albumin values which were determined in that experiment were also incorporated in this experiment. In addition, the absorbance readings which were used to calculate the absolute albumin values with species specific standards, were, in the case of ovine and equine, re-applied to the bovine albumin standard curve in order to provide albumin values which were derived from a non-specific standard.

ResultsTABLE 11.5SERUM ALBUMIN - AGAROSE GEL ELECTROPHORESIS AND THE 'IMMEDIATE'B.C.G. REACTION COMPARED, USING SPECIES SPECIFIC AND NON-SPECIFICSTANDARDS.

Species	No	Method	Std.	\bar{x} g/l	s	Paired 't' tests					Range g/l
						't'	p	df	$\bar{\Delta}$ g/l	Δs	
Equine	19	1	n/a	22.7	2.53	1.118	>0.2	18	0.44	1.72	18.9-28.1
	19	2	E	22.2	2.49						17.5-26.5
	19	1	n/a	22.7	2.53	2.359	<0.05	18	-1.50	2.58	18.9-28.1
	19	2	B	24.2	2.69						19.0-28.5

TABLE 11.6

Ovine	16	1	n/a	29.8	3.31	1.144	>0.2	15	0.36	1.26	21.1-35.5
	16	2	O	29.5	3.40						19.2-34.5
	16	1	n/a	29.8	3.31	15.620	<0.001	15	5.12	1.31	21.1-35.5
	16	2	B	24.7	2.80						16.3-28.7

TABLE 11.7

Bovine	19	1	n/a	30.8	2.74	0.897	>0.3	18	0.37	1.81	26.3-35.7
	19	2	B	30.4	2.53						25.4-34.2

For statistical definitions see page 73 .

Method 1 = Electrophoresis.

Standard (Std.) E = Equine

2 = 'Immediate' B.C.G. Reaction.

O = Ovine

n/a = not applicable.

B = Bovine

TABLE 11.8

SERUM ALBUMIN - VALUES COMPARED USING THE 'IMMEDIATE' B.C.G.

REACTION WITH SPECIES-SPECIFIC AND NON-SPECIFIC ALBUMIN STANDARDS.

Species	No	Method	Std.	\bar{x} g/l	s	Paired 't' tests					Range g/l
						't'	p	df	$\bar{\Delta}$ g/l	Δs	
Equine	19	2	E	22.2	2.49	-5.307	<0.001	18	-1.96	1.61	17.5-26.5
	19	2	B	24.2	2.69						19.0-28.3

TABLE 11.9

Ovine	16	2	O	29.5	3.40	-28.510	<0.001	15	4.77	0.67	19.2-33.7
	16	2	B	24.7	2.80						16.3-28.7

For statistical definitions see page 73 .

Method 2 = 'Immediate' B.C.G. Reaction.

Standard (Std.) E = Equine

O = Ovine

B = Bovine

TABLE 11.10

SERUM ALBUMIN - REGRESSION EQUATIONS FOR ELECTROPHORESIS
AND THE 'IMMEDIATE' B.C.G. REACTION COMPARED, USING SPECIES
SPECIFIC AND NON-SPECIFIC STANDARDS.

$$\underline{y \text{ (B.C.G.)} = m \times \text{(electrophoresis)} + c}$$

<u>Species</u>	<u>No.</u>	<u>Regression Equations</u>
Bovine	19	$y = 0.70 x + 8.75 \text{ (} r = 0.760 \text{)}$
Ovine	16	$y = 0.95 x + 1.08 \text{ (} r = 0.928 \text{)}$
Equine	19	$y = 0.76 x + 5.08 \text{ (} r = 0.764 \text{)}$

Using species specific albumin standard.

TABLE 11.11

Ovine	16	$y = 0.78 x + 1.47 \text{ (} r = 0.920 \text{)}$
Equine	19	$y = 0.54 x + 11.40 \text{ (} r = 0.519 \text{)}$

Using non-specific (bovine albumin standard).

For regression equation definitions see page 106.

Tables 11.5, 11.6 and 11.7 include some of the data which also appeared in Tables 11.1, 11.2 and 11.3. This data was included for ease of comparison with the additional information which the tables provide. In the tables, the mean and standard deviation are given, for albumin values in two species, by electrophoresis and by the 'immediate' B.C.G. reaction using non-specific (bovine) albumin standards. Paired 't' tests were carried out between equine and ovine electrophoretic albumin values and 'immediate' B.C.G. albumin values, calculated against the non-specific albumin standards. In these species, the statistical differences in albumin levels by the electrophoretic and the B.C.G. method

were $p = < 0.05$ and $p = < 0.001$ respectively; the mean absolute differences being -1.50 g/l and $+ 5.12$ g/l.

The regression equations for equine and ovine serum albumin by the 'immediate' B.C.G. reaction (y) (non-specific standard) and (x) electrophoresis, were formulated and are included in Table 11.11. (The regression equations in Table 11.10 are those from Table 11.4 (species specific standards) which have been included for comparison.)

Tables 11.8 and 11.9 show the mean and standard deviation for equine and ovine serum albumin by the 'immediate' B.C.G. reaction, using species specific and non-specific (bovine) albumin standards. The results of the paired 't' tests between the two procedures were: ovine $p = < 0.001$ and equine $p = < 0.001$ the mean difference being $+ 4.77$ g/l and $- 1.96$ g/l respectively.

Discussion

Before any statistical figures are discussed it must be acknowledged at this point, in light of a recent article by Altman (1980) that the incorrect choice of statistical method of analysis in comparison studies can lead to situations where some misinterpretation is likely. Altman stated that one common and completely misguided approach is to plot values obtained by each different method and then proceed to calculate the correlation coefficient. He pointed out that it is common to fail to appreciate what information the correlation coefficient provides, as it is possible, even with only moderate agreement, on a scatter diagram of individual values, to produce a high

correlation coefficient. This leads to the assumption by some that the two methods, in absolute terms, are in good agreement. Altman emphasised that, to test an observed correlation coefficient for statistical significance is really a double negative situation. If the correlation coefficient proves to be significant this means that under the hypothesis of no association, the two variables are, in fact, associated. Under certain circumstances the magnitude of the correlation largely reflects the spread of measurements.

Other examples given by Altman on the misuse of statistical methods, are problems with 't' tests and data which does not comply with the statistical assumption that the two sets of data come from the same populations which are Normal and have the same variance. It is also a common error to ignore the fact that two sets of measurements may relate to the same (or matched) individuals, in which case a paired 't' test should be carried out. The simplest approach to ascertaining how well two measures agree irrespective of whether any additional statistical evaluation is undertaken, is to calculate the mean difference and the standard deviation of the differences between two measurements for each subject. These values are a direct reflection of accuracy and precision (Altman, 1980).

For each species studied, my results (Tables 11.1, 11.2 and 11.3) show the mean and standard deviation of each group of albumin values obtained after electrophoresis and by the 'immediate' and 10 minute B.C.G. reactions. The mean difference and standard deviation of the differences in albumin values after electrophoresis

and by the two B.C.G. reaction times were also calculated and are included with the results of the paired 't' tests. Since some authors have used linear regression analysis to demonstrate the degree of comparability between electrophoresis and the 'immediate' B.C.G. method, I also calculated regression equations for the three species based on results by these two methods (Table 11.4). The information gained from these equations i.e. how close y (electrophoresis) is to x (B.C.G.), (based on an ideal slope of 1), together with the coefficient of correlation, provides an indication of the consistency of differences between values by the two methods. Analysis of the same data by paired 't' tests indicates whether or not these differences are statistically significant.

Linear regression analysis of my results from Table 11.4 demonstrated a good correlation between electrophoresis and the 'immediate' B.C.G. method, using species specific standards when albumin results from the three species were compared. Paired 't' test analysis of the same data (Tables 11.1, 11.2 and 11.3) showed that any differences, in absolute terms, were not statistically significant. On the other hand, paired 't' test analysis of electrophoretic results and those by the 10 minute B.C.G. reaction demonstrated differences in absolute terms which were highly significant ($p = < 0.001$).

Linear regression analysis of results from equine and ovine serum by electrophoresis and the 'immediate' B.C.G. method using non-specific (bovine albumin) standard, demonstrated a poor correlation (slope 0.54 and coefficient of correlation 0.519)

and a good correlation (slope 0.78 and coefficient of correlation 0.920) respectively. However when the same data was subjected to paired 't' test analysis (Tables 11.5 and 11.6) there were significant (equine $p = < 0.05$) or highly significant (ovine $p = < 0.001$) differences in absolute terms between individual values by the two methods. The latter case i.e. high correlation by highly significant differences between individual values, is similar to an example given by Altman (1980). Under certain circumstances, the magnitude of the correlation coefficient may largely reflect the spread of the measurements and a good correlation between methods does not necessarily indicate that individual values would agree in absolute terms, but merely that to some degree they are related.

I compared the statistically derived conclusions from my own work with those from previously published work. Gentry and Lumsden (1978) compared albumin levels (normal and abnormal subjects) in various animal species using agarose gel electrophoresis and the 'immediate' B.C.G. method. Using species specific standards they achieved an excellent correlation between the two methods, which was reflected in the magnitude of their coefficients of correlation; $r = 0.936$, $r = 0.888$, $r = 0.986$ and $r = 0.972$, for equine, bovine, porcine and canine serum respectively. They found that although there was a high degree of correlation, the absolute differences between the two methods were statistically significant (bovine $p = < 0.05$, porcine $p = < 0.01$ and canine $p = < 0.01$). Since these differences were consistent over the measurement range, the authors considered that this did not invalidate the reliability

of the B.C.G. method. Due to the fact that serum from each species was sampled from a mixture of normal and abnormal subjects, the measurement ranges were considerably wider than my ranges for normal animals (Tables 11.5, 11.6 and 11.7). For example Gentry and Lumsden quoted electrophoretic albumin ranges of 11-42 g/l, 12-35 g/l 16-39 g/l and 4-37 g/l for equine, bovine, porcine and canine serum respectively. Altman (1980) implied that in comparison studies, when subjects which were not of a totally Normal population, it was possible to achieve an enhanced correlation. My coefficients of correlation between the two methods are lower than those of Gentry and Lumsden; $r = 0.760$, $r = 0.764$ and $r = 0.928$ for bovine, equine and ovine serum respectively, in spite of the fact that, even in absolute terms, the differences between individual absolute values are not statistically significant ($p = >0.3$, $p = >0.2$ and $p = >0.2$).

Webster (1977), in his comparison of human albumin levels (normal and abnormal subjects) by cellulose acetate electrophoresis and the 'immediate' B.C.G. method also achieved an excellent correlation between the two, producing a regression equation of $y \text{ (B.C.G.)} = 1.008 \times x \text{ (electrophoresis)} + 2.817$ and a coefficient of correlation of 0.995. He also found that although there was a high degree of correlation, the differences in absolute values between the two methods were significant but consistent and noted that if 3 g/l was subtracted from each B.C.G. value, 50% of the sera gave the same result by both methods, 34% were within 1 g/l and 14% were within 2 g/l. (Although in effect this does indicate that 50% of B.C.G. values were at least 4-6 g/l higher than electrophoretic values.)

Gentry and Lumsden also carried out a comparison between electrophoretic and 'immediate' B.C.G. values in several species, using a non-specific (bovine) albumin standard. From the results of their linear regression analysis, they again achieved an excellent correlation between the two methods, over the measurement ranges previously described. The regression equations and coefficients of correlation for equine, porcine and canine serum respectively were $y \text{ (B.C.G.)} = 1.14 x \text{ (electrophoresis)} - 2.74$ and $r = 0.953$; $y = 1.192 x - 0.83$ and $r = 0.923$; $y = 0.876 x + 2.17$ and $r = 0.977$. With the exception of equine serum (in which apparently, the statistical significance was calculated between means and standard deviations) there were significant differences between individual absolute values by the two methods; porcine $p = < 0.01$ and canine $p = < 0.01$. No statistically significant differences between results were recorded in equine and porcine serum albumin by the 'immediate' B.C.G. reaction using species specific and non-specific (bovine) albumin standard curves to calculate the concentrations.

My linear regression results (Table 11.11) and B.C.G. results (Table 11.5) appear to be at variance with those of Gentry and Lumsden (1978). However for the reasons which I have outlined, it seems likely that the variations in linear regression results are mainly due to the widely differing populations utilised by these authors for statistical analysis. In view of the absorbance differences between equine and bovine albumin standard curves which I found and which are evident (Fig.5.1), I find it surprising that these authors found no statistically significant differences

between electrophoretic and 'immediate' B.C.G. albumin values when equine serum albumin concentrations were calculated from the bovine standard curve.

My results demonstrate that according to the procedures employed, there was a good correlation between the electrophoretic and 'immediate' B.C.G. methods provided species specific standards were used. This was also reflected in closely matching individual albumin values. It is recommended therefore that species specific standards should be used wherever possible, since the use of any other would be likely to cause discrepancy. This would certainly necessitate the use of a correction factor for each species, which in some cases such as ovine, may be considerable.

Binding of B.C.G. dye by albumin. It is clear from the results of these and other experiments that equivalent amounts of pure albumin from each species bind fixed amounts of B.C.G. dye to different degrees. This is reflected in the magnitude of absorbance produced at 628 nm when albumin is reacted with B.C.G. working dye reagent. In quantitative analysis therefore, discrepancies can occur when the absorbance produced by a standard amount(s) of pure albumin from one species is used to calculate the serum albumin concentration of another species. The degree of discrepancy is related to the degree of dissimilarity between the species specific and non-specific absorbance/concentration curves for pure albumin, (see Fig. 5.1). Human and bovine albumin curves are very similar, but ovine and equine albumin curves are markedly different from each other and noticeably different to the human and bovine curves. These differences become more pronounced as the albumin

concentration increases, with wide differences in absorbance values between the equine and ovine curves being evident.

The amino acid sequences and covalent structures of human and bovine albumin molecules were determined by Brown (1976) and these have been shown to be composed of a single polypeptide chain grouped into a series of nine disulphide bonded loops, which appear to associate into three separate domains, each with separate functions. The reasons for the differences in affinity for B.C.G. which each species of albumin exhibits are not certain, but based on observations by Peters (1977), a probable explanation could be that many molecules of the dye are loosely bound and that there are few specific and clearly identifiable binding sites for the dye on the albumin molecule. Peters noted that the relative binding of B.C.G. by various fragments of bovine albumin, isolated after limited proteolysis, was roughly proportional to the size of the fragment. A possible exception to this was observed in the region of loops 4-5 on the molecule. This is the region proposed as the bilirubin binding site and it is interesting to note that Webster (1977) found that increased levels of bilirubin did not influence the albumin levels determined by the B.C.G. reaction. It is probable that the differences in primary structure of the albumin molecule between species and the combination of the few specific binding sites for the dye, accompanied by loose electrostatic binding (protein anions with dye cations), produce binding variables between species. These variables have the net effect of giving a relatively high B.C.G. dye binding capacity and associated high absorbance values in some cases such as with equine albumin and the converse with others such as ovine albumin.

As was evident from the magnitude of the differences in serum albumin values between the 'immediate' and 10 minute B.C.G. reactions, some other proteins do react with the dye to produce absorbance changes. Webster, (1974) and Gustafsson, (1976) have shown that the major contributory protein components which act in this way are to be found in the electrophoretic alpha and beta globulin zones, but unlike albumin these do not react with the dye on a weight for weight basis. In view of the fact that loose electrostatic attraction with the dye is thought to account for at least part of the total binding capacity of albumin (Peters, 1977), it is probable that other anionic proteins such as alpha and beta globulins behave in a similar manner. Webster (1974) noted that alpha globulin (which is the fastest anodally migrating globulin, having a greater net negative charge than the others) produced only 20% of the colour intensity compared to an equivalent weight of albumin. Beta globulin (slower anodally migrating with less net negative charge) produced only 10% of the colour intensity compared to an equivalent weight of albumin. Gamma globulin on the other hand, which migrates only very slightly anodally, or slightly cathodally in certain species and on certain media, (due to net neutral or slightly net positive charge) did not react with B.C.G. dye to produce any increase in absorbance.

In the light of the above facts I have formulated the hypothesis that if loose electrostatic binding is responsible for part of the absorbance increase when albumin is reacted with B.C.G. working dye solution, then it is probable that the same happens with other anionic proteins (alpha and beta globulins), with the degree of

interaction being related to the net charge on particular protein molecules. The fact that gamma globulin is said not to react with the dye to produce any absorbance change, tends to support the hypothesis, since the gamma globulin(s) molecule is considered as having essentially a net neutral charge. This observation will be tested in the next experiment.

CHAPTER XII

EXPERIMENT XREACTION OF ALBUMIN-FREE SERUM AND GAMMA GLOBULIN PREPARATIONS
WITH B.C.G. WORKING DYE REAGENT.Introduction

The results of previous experiments have demonstrated that in each of the species studied, albumin values obtained by the 10 minute B.C.G. method were higher than when the 'immediate' reaction time was employed. The reason for the higher values has been attributed to a combination of interactions with the dye by other non-albumin material present in the serum, which also produce a change in absorbance. I have quoted authors (see page 39) who investigated the proteins mainly responsible for this in human serum. These appeared to be alpha globulins and to a lesser extent beta globulins. Gamma globulins did not appear to react with B.C.G. dye.

The purpose of this experiment is to obtain albumin-free serum from three species and to react this with B.C.G. working dye reagent to ascertain whether or not the total globulin fractions in each species will produce any absorbance change. The reaction of pure gamma globulin preparations will indicate whether or not this fraction could contribute to any absorbance change with B.C.G. dye. The albumin-free serum will be subjected to biuret total protein analysis and agarose gel electrophoresis, to determine the relative and absolute amounts of alpha, beta and gamma globulins present. From the results of these experiments, should the albumin-free serum react with the dye to produce a change in absorbance, it should be possible to gauge which globulin fraction(s) are likely to be responsible.

Materials and Methods

Preparation of albumin-free serum was carried out according to the procedure described on page 69. 20 ml of pooled serum from each species was processed and the first effluent collected from the affinity chromatography column was re-applied to ensure that possible albumin contamination would be minimal. During the final concentration procedure, biuret analysis of each albumin-free effluent was carried out until a total protein level of 30-35 g/l was reached in each case. The electrophoretic and biuret procedures are described on pages 64 and 61 .

Gamma globulin of each species was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. The purities of each were:- bovine 95%, ovine 90% and equine 98%. Gamma globulin preparations of 20 g/l and 40 g/l were prepared for each species by dissolving 0.1g and 0.2g respectively in 4 ml of NaCl and making up to volume in a 10 ml volumetric flask. Since each of these solutions was slightly turbid, it was necessary to perform appropriate blank corrections in the B.C.G. analysis with succinate buffer, to compensate for any absorbance change due to turbidity.

ResultsTABLE 12.1

RELATIVE AND ABSOLUTE AMOUNTS OF GLOBULINS PRESENT IN THE
ALBUMIN-FREE SERUM.

Species	Total Globulin	Alpha-1 Globulin		Alpha-2 Globulin		Beta Globulin		Gamma Globulin	
	g/l	%	g/l	%	g/l	%	g/l	%	g/l
Bovine	±34.0	8.0	2.7	8.0	2.7	11.5	3.9	71.5	24.4
Ovine	*33.0	0.5	0.2	9.0	3.0	12.5	4.1	75.0	24.7
Equine	33.0	15.9	5.2	24.2	8.0	25.5	8.4	34.4	11.4

± - includes 1% albumin contamination.

* - includes 3% albumin contamination.

TABLE 12.2

REACTION OF VARIOUS CONCENTRATIONS OF GAMMA GLOBULIN PREPARATIONS
WITH B.C.G. WORKING DYE REAGENT ('IMMEDIATE' AND 10 MINUTE REACTIONS).

Bovine : No measurable reaction with 20 g/l or 40 g/l preparations.

Ovine : " " " " " " " " " "

Equine : " " " " " " " " " "

TABLE 12.3

REACTION OF ALBUMIN-FREE SERUM WITH B.C.G. WORKING DYE REAGENT
('IMMEDIATE' AND 10 MINUTE REACTIONS).

Species	'Immediate' Reaction	10 Minute Reaction
	g/l	g/l
Bovine	± 0.9	± 3.3
Ovine	* 1.2	* 3.0
Equine	0.8	3.4

± - figure includes 0.3 g/l albumin contamination.



* - figure includes 1.0 g/l albumin contamination.

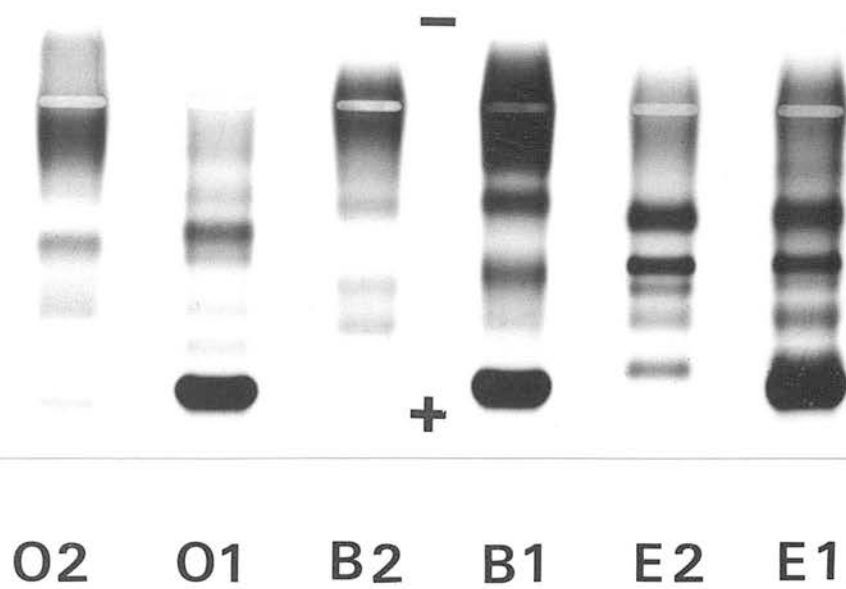
FIGURE 12

ELECTROPHORESIS OF WHOLE AND ALBUMIN-FREE SERUM.

KEY

- O2 - Ovine albumin-free serum.
- O1 - Ovine whole serum.
- B2 - Bovine albumin-free serum.
- B1 - Bovine whole serum.
- E2 - Equine albumin-free serum.
- E1 - Equine whole serum.
- a + b - alpha and beta globulins.


a + b




The effectiveness of the affinity chromatography procedures in removing albumin from the pooled serum is shown in Fig. 12. The maximum contamination with albumin was 3% in the ovine sample. Values were calculated from the electrophoretic scans of these patterns, by relating each percentage value to the appropriate globulin value. These absolute values are presented in Table 12.1.

The results of reacting purified gamma globulin preparations and albumin-free serum with B.C.G. working dye solution are given in Tables 12.2 and 12.3 respectively.

Discussion

After collection and concentration of the albumin-free serum, in each case a few millilitres of clear fluid was obtained, which exhibited a slight yellow/brown pigmentation. From Fig. 12 it can be observed that removal of albumin was successfully achieved. Only minimal amounts of albumin contamination, 3% in O2 and 1% in B2 was detected (calculated from the densitometric trace of each). The slight contamination encountered was probably due to either insufficient contact time between the pooled whole serum and the affinity gel, or that the gel itself had deteriorated slightly after repeated regeneration. The latter explanation seems more likely since the same procedure was followed for each batch of serum processed and the first serum (equine) showed no detectable albumin contamination after electrophoresis.

It is interesting to note that in Fig. 12, a well defined alpha globulin zone is visible in E2, closest to the anode. This is the zone which I have chosen to term alpha-la

and this will be discussed in the next experiment. Since this globulin zone migrates on the cathodal shoulder of the albumin zone it is often very difficult to differentiate when a serum protein densitometric pattern is examined, but in the absence of albumin it is clearly demonstrated to be in this position. The relative proportions of equine alpha and beta ($\alpha + \beta$) globulins appear to be greater than in the other two species both before and after processing, whereas the relative proportion of gamma globulin appears to be less. The processing and concentration of the pooled sera appears to have had a different effect on the bovine and ovine globulins. It might have been expected to find more intense alpha and beta bands in B2 and O2 after concentration, but it seems that some globulins may have been lost at some stage of the processing.

Biuret analysis and densitometric scanning of each of the albumin-free electrophoretic profiles allowed the relative and absolute amounts of globulin in the major zones to be calculated (Table 12.1). Although the total globulin concentrations of each albumin-free serum are almost identical, the relative and absolute amounts of globulin between each major zone vary considerably in some cases.

Since the various preparations of gamma globulins were found not to react with B.C.G. working dye solution (Table 12.2), any possible contribution to the reactions observed in Table 12.3 by this fraction can be discounted. These findings are in agreement with those of Webster (1974) and Gustafsson (1976). Webster also found that although the absorbance was relatively small compared to that produced by albumin, human serum albumin produced twice the absorbance

with B.C.G. dye than equivalent amounts of beta globulin.

It is probable therefore that in animal serum alpha globulins would be likely to contribute similarly in such a reaction, although from the results in Table 12.1 and Table 12.3 it would appear that there are certain species differences in reactivity. It is evident for example, that equine albumin-free serum contains more than four times as much alpha globulin and twice as much beta globulin as its ovine counterpart and three times as much alpha globulin and twice as much beta globulin, as the bovine albumin-free serum. This however is not reflected in a correspondingly greater proportion of reactivity with the dye. It would appear that the overall reactivity (after correction) of bovine and ovine globulins are not dissimilar. The beta globulin concentrations were close in absolute terms and ovine albumin-free serum contained only minute amounts of alpha-1 globulin which would be unlikely to have measureable effect on the reaction in Table 12.3. Although the total ovine alpha globulin concentration (mostly alpha-2) was 40% less than its bovine counterpart, this was matched by a similar decrease in reactivity.

From the results of these experiments it seems that although the concentration of alpha and beta globulins is a factor in determining the magnitude of their reaction with the dye, since these globulins do not react on a weight for weight basis, increasing the concentration of each of these would produce some positive effect on absorbance, but would be unlikely to produce any linear absorbance/concentration relationship with the dye as does albumin. It would seem that reactivity is a greater factor than concentration. It has already been stated

that equine albumin-free serum contained much more alpha and beta globulin than bovine, yet apparently the reaction with B.C.G. working dye was almost identical. This could indicate that the concentration of a specific bovine protein(s) is greatly in excess of its equine counterpart and is not immediately obvious, which seems unlikely in view of the much lower corresponding globulin levels of each main fraction, or that the reactivity of certain bovine globulin(s) are greater than their closest equine counterparts. The latter explanation may be one reason for the earlier differences noted between the 'immediate' and 10 minute B.C.G. serum albumin values, where those of bovine were almost twice those of equine.

Further studies on the reactivity of purified bovine, ovine and equine alpha and beta globulins would provide some more positive evidence as to whether or not this was the case. It would also be of interest to ascertain if raised serum levels of certain alpha and beta globulins, or specific members of these, as found in human serum, would cause greater over estimation of albumin by the 10 minute B.C.G. reaction.

CHAPTER XIII

EXPERIMENT XI

CALCULATION OF Rf VALUES OF BOVINE, OVINE AND EQUINE SERUM PROTEINS AFTER AGAROSE GEL ELECTROPHORESIS.

Introduction

The main objective of this work was to compare serum albumin levels by a dye binding technique with those obtained after agarose gel electrophoresis. Since electrophoresis yields information not only on albumin but also on the main globulin fractions and in some cases sub-fractions, I decided to take this opportunity to employ some of the additional data which I had gathered during the course of the comparative exercise and use it to calculate a reference range of electrophoretic Rf values for this laboratory. It is generally accepted that each laboratory should determine its own normal values for protein electrophoresis in each animal population to be studied, since normal ranges often show quite wide variations which are largely attributable to differences in separating techniques and quantitating procedures. (Liberg, 1977a).

Materials and Methods

Electrophoretic data mainly from the densitometric traces in Experiments III and VIII, was used to calculate the Rf values of various globulin fractions in bovine, ovine and equine serum. The equine serum used in Experiment VII was also subjected to electrophoresis as described on page 64. The procedures for serum protein recording, annotation and calculation of Rf values was followed as described on page 65. The breeds and age groups of the animals were as follows:-

- Bovine - 2-10 y.o. Blue-grey heifers and cows. (32)
- Ovine - 18 m.o.-2y.o. Greyface gimmers. (20)

Equine A - 2-6 y.o. Eriskay ponies. (25)

Equine B - 2-4 y.o. Thoroughbred horses. (10)

Results

TABLE 13

Rf VALUES OF BOVINE, OVINE AND EQUINE SERUM PROTEINS AFTER
AGAROSE GEL ELECTROPHORESIS.

Species	Bovine (32)		Ovine (20)		Equine A (25)		Equine B (10)	
Fraction	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s
Albumin	1.000	-	1.000	-	1.000	-	1.000	-
Globulins								
Alpha-1	0.791	0.015	0.767	0.016				
Alpha-1a					0.890	0.040	0.890	0.037
Alpha-1b					0.770	0.022	0.770	0.030
Alpha-2	0.599	0.014	0.472	0.022				
Alpha-2a					0.652	0.022	0.630	0.015
Alpha-2b					0.560	0.041	0.560	0.050
Inter alpha/ beta	0.455	0.016						
Beta			0.273	0.110				
Beta-1	0.297	0.015			0.341	0.033	0.370	0.036
Beta-2	0.147	0.008			0.210	0.023	0.180	0.024
Gamma	n/a	-	n/a	-	n/a	-	n/a	-
Gamma-2			-0.256	0.024				

n/a = not applicable.

After electrophoresis and densitometric scanning, each pen trace was examined individually. The mean electrophoretic Rf values obtained are set out in Table 13.

In bovine serum, an albumin zone and six globulin zones were clearly visible in every case. (See Fig. 13.1). These were

FIGURE 13.1

The main globulin peaks detectable in bovine serum after agarose gel electrophoresis.

- 1 - Alpha-1 globulin.
- 2 - Alpha-2 globulin.
- 3 - Inter alpha/beta globulin.
- 4 - Beta-1 globulin.
- 5 - Beta-2 globulin.
- 6 - Gamma globulin.

FIGURE 13.2

The main globulin peaks detectable in ovine serum after agarose gel electrophoresis

- 1 - Alpha-1 globulin.
- 2 - Alpha-2 globulin.
- 3 - Beta globulin.
- 4 - Gamma-1 globulin.
- 5 - Gamma-2 globulin.

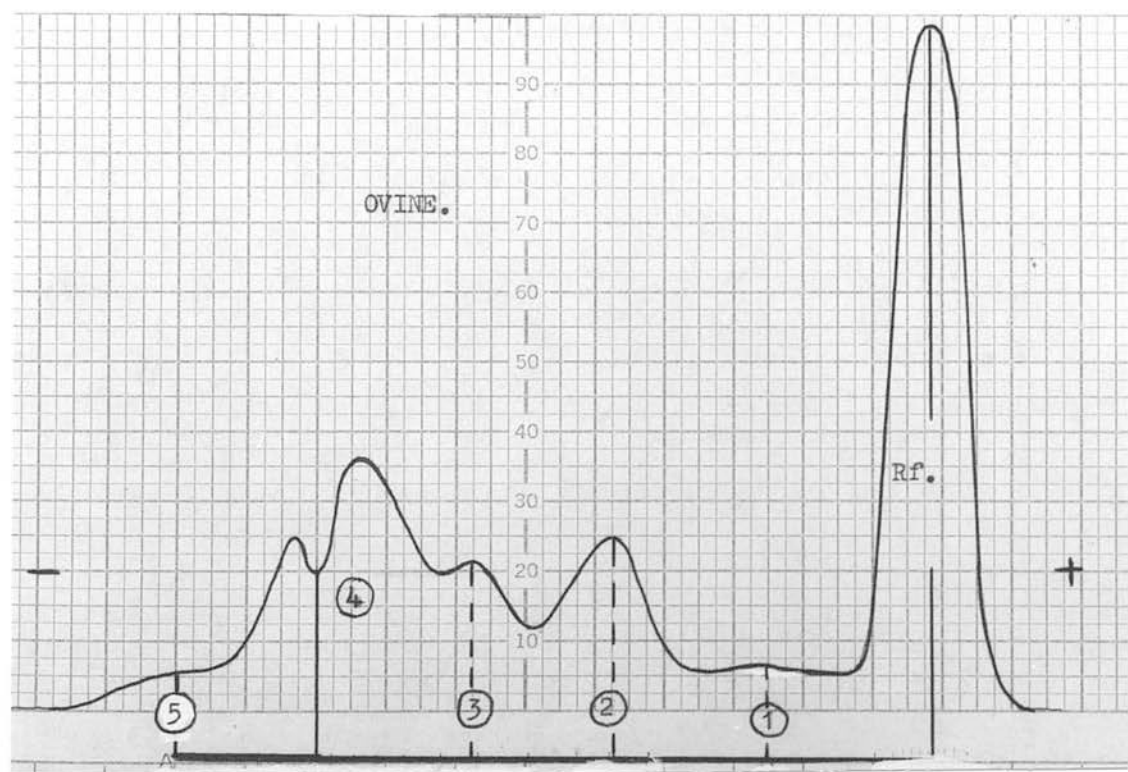
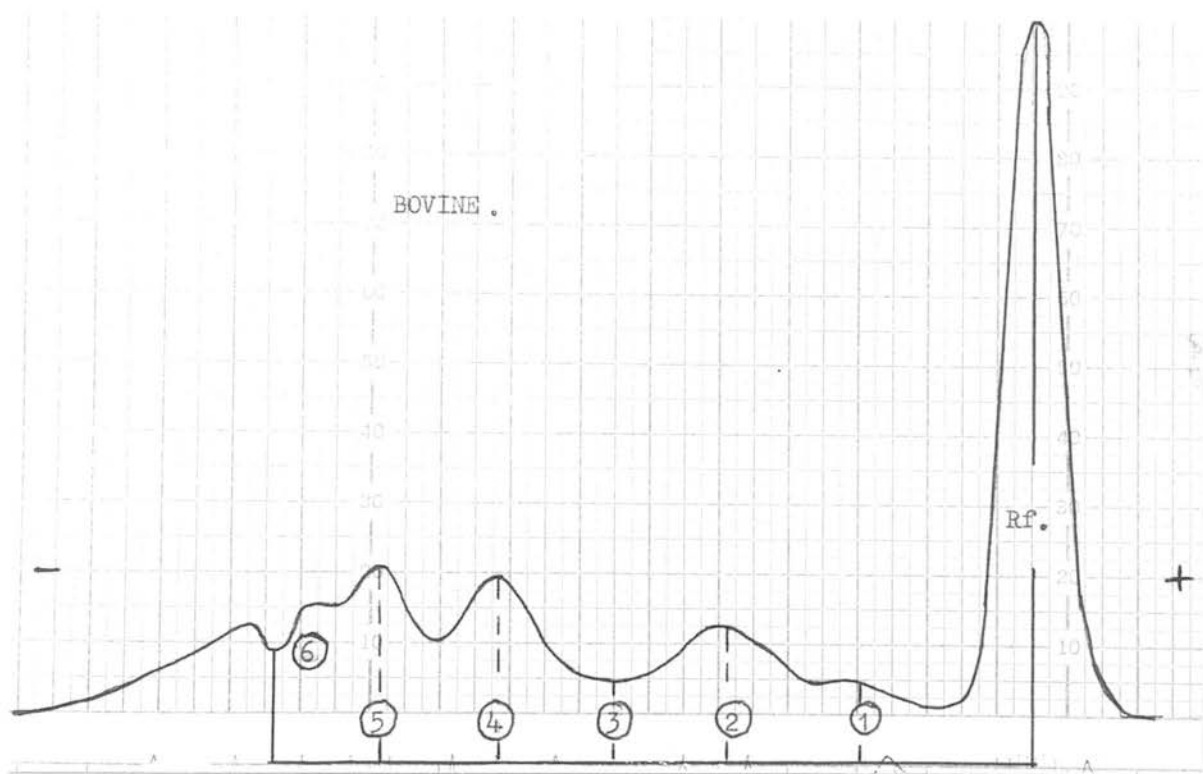
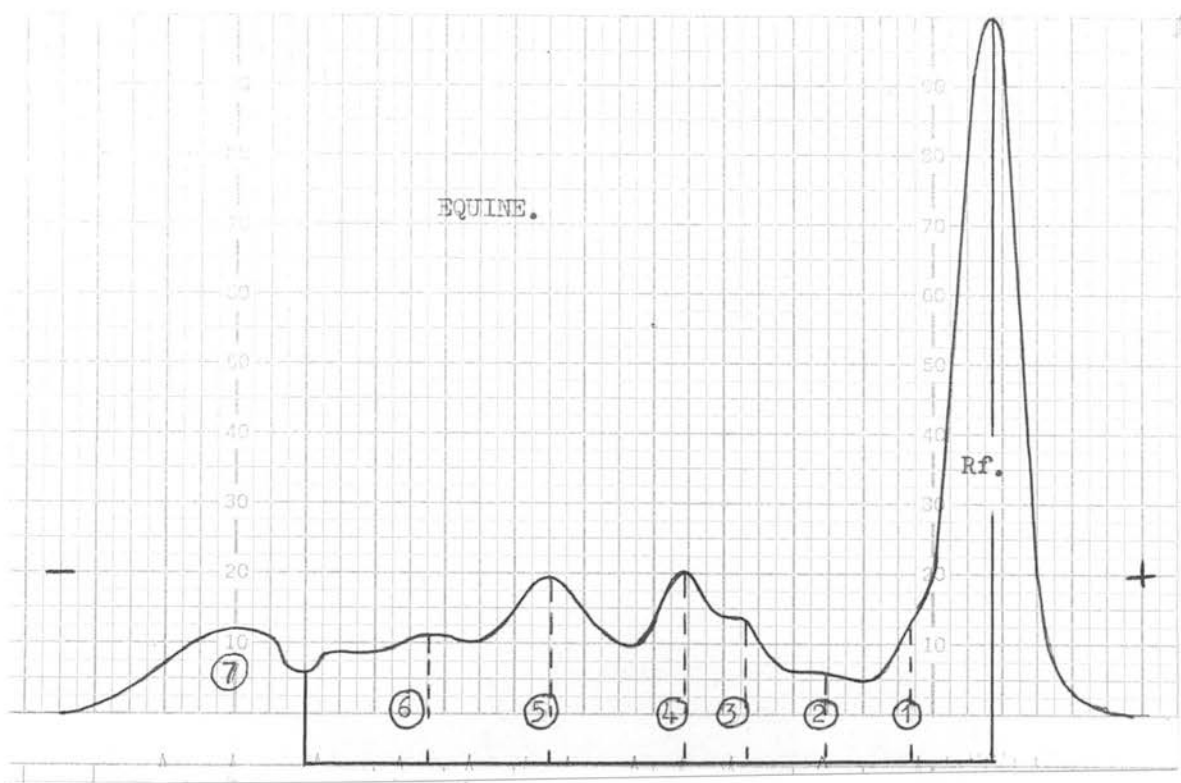


FIGURE 13.3

The main globulin peaks detectable in equine serum after agarose gel electrophoresis.

- 1 - Alpha-1a globulin.
- 2 - Alpha-1b globulin.
- 3 - Alpha-2a globulin.
- 4 - Alpha-2b globulin.
- 5 - Beta-1 globulin.
- 6 - Beta-2 globulin.
- 7 - Gamma globulin.



designated, in order of decreasing mobility, albumin, alpha-1 globulin, alpha-2 globulin, an inter alpha/beta region, beta-1 globulin, beta-2 globulin and gamma globulin.

In ovine serum, an albumin zone and five globulin zones were clearly visible (See Fig. 13.2). These were designated albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, gamma-1 globulin and gamma-2 globulin. The gamma-2 globulin was unusual in that its peak was found to be on the cathodal side of the application slit and hence was given a negative (-) mobility figure.

In each of the two groups of equine sera i.e. ponies and horses, an albumin zone and at least seven globulin zones were clearly visible in each case (See Fig. 13.3). These were designated albumin, alpha-1a and alpha-1b globulins, alpha-2a and alpha-2b globulins, beta-1 globulin, beta-2 globulin and gamma globulin. In some cases sub-fractionation of the beta-globulins was just possible, but since these were so few it was decided not to calculate figures for inclusion in the table. The alpha-1a globulin region was unlike the alpha-1 region in the other two species in that it was located on the cathodal shoulder of the albumin peak.

In Figs. 13.1, 13.2 and 13.3 the gamma globulin zones have been numbered differently to those of the other globulins. Since lines were drawn through the sample application point (appears as indentation) and not the actual gamma peak the zone number of each has been displaced from the other to avoid misinterpretation.

Discussion

In each of the species studied the relative migration of each serum protein zone was distinctive (see Figs. 13.1, 13.2 and 13.3).

With a little experience the species of the animal could be fairly easily ascertained after only a brief inspection of the densitometric trace e.g. after electrophoresis, bovine serum exhibited a characterisitic, fairly broad inter alpha/beta region, ovine serum exhibited a gamma-2 globulin peak which was located on the cathodal side of the application slit and equine serum exhibited an alpha-la region on the cathodal 'shoulder' of the albumin peak. This 'shoulder' was recognised by Kristensen and Firth (1977) who did not term the region alpha-la, but alpha-l. They did however point out that there was disagreement on the nomenclature of equine serum protein fractions and stated their reasons for their adopted nomenclature of the alpha globulin sub-fractions. They found that since the next fraction to alpha-l globulin demonstrated 3 or 4 distinct features in some cases, it was decided to term these, where applicable, alpha-2aa, 2ab, 2b and 2c globulins.

I decided not to follow this nomenclature when classifying the alpha globulin sub-fractions of equine serum. Basing my hypothesis on the results of much research on individual proteins in the human fields, the generally accepted globulin component which has the fastest electrophoretic mobility and which would be of a level likely to make a measurable contribution in this region is alpha lipoprotein, followed by alpha-l antitrypsin and to a lesser extent, alpha-l acid glycoprotein (orosomuroid). Since each of these proteins are classified as alpha-l globulins (Laurell et al. 1978), it is probable that my equine alpha-la region corresponds to the major proportion of the alpha lipoprotein component. The alpha-lb region corresponds to the remainder of this, together

with the other two alpha-1 protein components. If this is the case, the two alpha-2 fractions according to my classification would be likely to be composed of the next fastest migrating protein components, namely alpha-2 macroglobulin and haptoglobin. Although there was generally a wider variation in my figures (much lower population and higher standard deviation) than those of Kristensen and Firth, the mean Rf values for most of the major fractions were in agreement. Direct comparison of the Rf values in the alpha globulin region was not possible owing to the differing nomenclature and the further sub-fractions delineated by these authors, but if their mean alpha-1 value is compared with my mean alpha-1a value for horses, the difference in Rf value between the two sets of figures is +0.039 (+3.9%). If their alpha-2aa/2ab mid value is compared with my mean alpha-1b value, the difference is -0.002 (-0.2%). If their mean alpha-2b value is compared with my mean alpha-2a value, the difference is +0.002 (+2%) and if their mean alpha-2c value is compared with my mean alpha-2b value, the difference is -0.009 (-0.9%).

Although Osbaldiston (1972) did not quote absolute figures for Rf values in his study of the serum protein fractions in domestic animals, inspection of his diagram of their relative migration distances suggests that there would be a less favourable comparison of corresponding Rf values. Unlike the present study and that of Kristensen and Firth (1977), Osbaldiston used cellulose acetate support medium in his electrophoretic survey and in general demonstrated less protein zones with a comparatively wide Rf range in each. In bovine serum he demonstrated albumin and four

globulin zones. The alpha globulin zone was not differentiated and no recognition was given to an inter alpha/beta region. Liberg (1977a) did not quote Rf values for bovine serum after agarose gel electrophoresis on water cooled plates, but did however recognise an inter alpha/beta region and also reported a similar number and distribution of globulin zones as was found in this study. Carlstrom and Liberg (1975) had previously demonstrated a lipoprotein band using lipid staining in the inter alpha/beta region.

Ek (1970) included the sera from 30 normal sheep in his electrophoretic study of serum proteins in certain species. Using cellulose acetate membrane as support medium he quoted relative percentage figures for albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin. It was interesting to note that he was not able to differentiate beta-globulin, whereas Osbaldiston (1972) was, using essentially the same media with a slightly different pH and buffer. Rf values were not calculated in the study. Although Ek did recognise a second small gamma globulin peak in ovine serum, he did not quote any figures for this, whereas Osbaldiston both recognised and quoted figures for gamma-2 globulin.

As seen in Fig. 13.2, I was able to demonstrate albumin and five other globulin peaks in ovine serum after agarose gel electrophoresis. The actual peak of what is apparently gamma-1 globulin lies on the anodal side of the sample application slit, which is used as the zero reference point for calculating the Rf values. The gamma globulin region in the ovine serum protein densitometric trace appears to have a much broader base than the gamma region

in the other two traces. It is possible that since ovine serum is apparently the only species not universally demonstrating two beta globulin peaks, either the overall beta globulin components in adult sheep serum cannot be differentiated because of their low concentration and close relative mobilities, or because a second small beta peak of lesser mobility may be masked by the overall width and magnitude of the gamma region. The findings of Knight and Leek (1973) would tend to support the former hypothesis, in that their results from a cellulose acetate electrophoresis study of the serum protein fractions in lambs, which were sampled soon after birth and at fortnightly intervals up to 12 weeks, showed that the beta-2 peak, which they were able to demonstrate, gradually diminished as the age of the lamb increased.

Like Osbaldiston (1972) I found that a gamma-2 peak was clearly identifiable in ovine serum, but I was not able to differentiate beta globulin as previously discussed and this was consistent with the results of Ek (1970).

As a result of this comparison, it would appear that the recommendations quoted in the introduction to this chapter are sound. The best comparison i.e. present study vs. Kristensen and Firth (1977) was obtained using the same support media and conditions and a comparable densitometric scanning instrument.

CHAPTER XIV

PROTEIN LEVELS IN NORMAL BOVINE, OVINE AND EQUINE SERUM.EXPERIMENT XII

ALBUMIN VALUES BY B.C.G. 'IMMEDIATE' REACTION.

Introduction

During the course of this work I gained access to blood samples from several specific groups of normal cattle, sheep and horses (see Table 14.1). It is proposed to use the serum obtained from these to establish laboratory reference values for albumin in each of the species stated, by the 'immediate' B.C.G. reaction.

It has already been shown that serum albumin results obtained following this procedure are in good agreement with a reference technique for serum albumin and also with albumin figures by agarose gel electrophoresis. (See Chapters X and XI). Since results using the 'immediate' B.C.G. reaction are clearly more specific for albumin than by the 10 minute reaction, it is necessary to establish some new reference levels to ensure that valid comparisons may be made if the shorter reaction time is employed.

Materials and Methods.

Blood samples were obtained from a total of nine groups of healthy animals from the three species. The samples were collected in a similar manner to that described in Chapter VI and after separation, any serum not analysed on the day of collection was stored at -15°C for no longer than 7 days.

The sera from each species was analysed for albumin by the modified B.C.G. dye binding procedure, using the 'immediate' reaction, as described in Chapter VIII. Albumin concentrations were calculated from each serum/dye absorbance reading at approximately 20 seconds,

against albumin standard serum/dye absorbance readings taken after the same time interval. Species specific albumin standards were employed with each group of sera analysed.

Results

TABLE 14

SERUM ALBUMIN LEVELS IN NORMAL ANIMALS BY THE 'IMMEDIATE' B.C.G. REACTION.

Species	No.	Serum albumin g/l		Range g/l
		\bar{x}	s	
Bovine (A)	8	28.5	2.18	25.4 - 32.1
Bovine (B)	16	31.3	1.85	28.6 - 34.2
Bovine (C)	8	33.9	2.54	30.5 - 37.5
Bovine (D)	8	33.3	1.90	31.8 - 37.2
Bovine (E)	10	20.4	1.40	17.8 - 22.1
Equine (A)	25	22.3	3.21	18.6 - 26.5
Equine (B)	10	27.8	2.13	25.2 - 31.9
Ovine (A)	8	30.1	3.53	22.8 - 34.0
Ovine (B)	8	28.8	2.86	23.0 - 30.8

Breeds and age groups.

Bovine (A) - 2 y.o. Blue-grey heifers.

Bovine (B) - 5-10 y.o. Blue-grey cows. (Mid. pregnancy).

Bovine (C) - 10 y.o. Blue-grey cows, calved. (2 months).

Bovine (D) - 4 y.o. Friesian cows, calved. (6 months).

Bovine (E) - 3-4 w.o. Ayrshire calves.

Equine (A) - 2-6 y.o. Eriskay ponies.

Equine (B) - 2-4 y.o. Thoroughbred and Thoroughbred cross horses.

Ovine (A) - 18 m.o. Greyface gimmers.

Ovine (B) - 2 y.o. Greyface gimmers. (Pre-parturition).

The mean and standard deviation of albumin values were calculated from various breeds and age groups of the three animal species and are set out in Table 14.

Discussion

Serum albumin levels are presented from a total of nine groups of healthy animals. These levels, obtained using the 'immediate' B.C.G. reaction are lower than would be reported if a longer reaction time was used (as shown in Experiment IV).

Apart from two groups of animals in Table 14, calves and ponies, the mean values for the other groups were within the relatively narrow range of 27-34 g/l. Age related albumin levels in calves and breed and nutritionally related levels in ponies have been observed by Kelly and Nutter (personal communication) and these observations offer at least a partial explanation for the lower values which I found.

In Table 14 the highest albumin values were found in bovine groups C and D, which included cows in their second and third months of lactation. Rowlands (1978) showed that dairy cows in their second and third months of lactation had serum albumin levels approximately 3.0 g/l higher than their mid-pregnancy levels. I found a similar difference in blue grey cows at mid-pregnancy and 2 months after calving.

Although cattle breeds and ages were not identical, if some of my figures from Table 14 are examined with those from Table 4.2b page 47-, certain comparisons can be made. The albumin values quoted by Liberg (1977a) for 25 Swedish Red dairy cows, using the B.C.G. method of Doumas et al. (1971) and employing a 10 minute reaction

time are 7.0 g/l higher than my figures for Friesian cows by the 'immediate' B.C.G. reaction. This positive difference is greater than my difference of 4.3 g/l (see Table 7.1a, page 84) when bovine serum albumin values by the 'immediate' and 10 minute B.C.G. reactions were compared. This difference could probably be at least partly due to breed, since Tumbleson et al. (1972) recorded certain breed related differences in albumin levels. Their figures show that mean albumin values for Holstein cows were 2-3 g/l higher than in Guernsey cows.

Matthews (1980) (personal communication) used the B.C.G. method of Doumas et al. in his study of certain serum proteins in 48 normal horses and ponies. The 10 minute B.C.G. reaction was employed, but the mean albumin figure of 28.9 ± 3.8 g/l was slightly higher than my mean figure of 27.8 ± 2.13 g/l for horses, using the 'immediate' B.C.G. reaction. The actual ratio of horses to ponies in Matthews study is not known at this time, but according to my figures for ponies (Table 14) and the information gained from Kelly and Nutter (unpublished data), the inclusion of ponies would be likely to lower the mean albumin value in the mixed group.

Khalaf (1977) also used the B.C.G. method of Doumas et al. in his determination of ovine serum albumin. His pre-parturition albumin figures for ewes, employing the 10 minute B.C.G. reaction were approximately 5 g/l lower than my pre-parturition albumin figures for gimmers using the 'immediate' B.C.G. reaction. According to unpublished data from this laboratory, the breed and age differences between these animals would not account for the differences between our results. It is known however (personal communication)

that Khalaf did not use species specific albumin standards in his B.C.G. analysis, owing to non-availability, and he substituted these with bovine albumin standards. I have shown in Experiment IX that the use of non-specific albumin standards to calculate serum albumin concentrations, especially in ovine species, can lead to normal range discrepancies of approximately 5 g/l. It seems likely therefore, that this is the major reason for the differences between my results and those of Khalaf.

CHAPTER XV

EXPERIMENT XII

TOTAL PROTEIN AND ELECTROPHORETIC VALUES.

Introduction

For reasons outlined in the introduction to the previous experiment, it is proposed to establish laboratory normal values for total protein and the various electrophoretic protein fractions in each of the species.

As can be seen in Tables 4.2 to 4.4 (pages 46 to 51) there are marked variations in electrophoretic values between species, especially when different types of support media are used. By clearly defining the breeds and ages of the various animal groups used in this and other experiments it is anticipated that a useful set of normal values will be compiled which may be used in future comparisons in this laboratory.

Materials and Methods

The sera used in this experiment were similar to those used in the previous experiment and the methods used are described on pages 61 and 64 .

Results

TABLE 15.1

AGAROSE GEL ELECTROPHORETIC SEPARATION (DISTRIBUTION %) OF ALBUMIN AND GLOBULINS IN NORMAL

BOVINE SERUM. (Mean \pm standard deviation.)

Group	No.	Albumin (%)		Globulin fractions (%)											
				Alpha-1		Alpha-2		Inter Alpha/Beta		Beta-1		Beta-2		Gamma	
\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s		
A	10	37.7	4.30	7.9	2.34	14.3	2.46	4.3	0.77	19.1	1.72	8.9	2.18	9.7	4.7
B	8	46.8	2.78	3.4	0.62	8.3	0.71	4.0	0.53	11.8	1.18	11.3	1.91	14.4	1.89
C	8	48.3	3.70	2.0	0.79	8.1	0.70	2.8	0.47	9.1	0.52	10.5	0.93	20.1	2.18
D	8	45.9	1.89	2.1	0.53	7.5	1.13	3.4	0.45	10.1	1.83	11.2	1.61	20.3	2.13
E	8	50.3	3.17	1.8	0.50	7.4	0.56	2.9	0.70	8.2	0.68	10.8	1.50	18.4	2.46
F	8	51.0	4.66	3.1	0.92	8.5	1.46	3.5	0.38	9.9	1.20	8.6	1.12	16.2	3.96

Group A = 3-4 w.o. Ayrshire calves.
 B = 2 y.o. Blue-grey heifers.
 C = 5 y.o. Blue-grey cows, in calf.
 D = 10 y.o. Blue-grey cows, in calf.
 E = 10 y.o. Blue-grey cows, calved 2 months.
 F = 4 y.o. Friesian cows, calved 3 months.

TABLE 15.2

AGAROSE GEL ELECTROPHORETIC SEPARATION (DISTRIBUTION %) OF ALBUMIN AND GLOBULINS IN
 NORMAL OVINE SERUM. (Mean \pm standard deviation.)

Group	No.	Albumin (%)		Globulin fractions (%)									
				Alpha-1		Alpha-2		Beta		Gamma-1		Gamma-2	
		\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s
G	20	38.0	4.31	6.3	1.72	11.4	1.20	7.5	1.67	29.1	4.52	7.1	2.36

Group G = 1½-2y.o. Greyface gimmers.

TABLE 15.3

AGAROSE GEL ELECTROPHORETIC SEPARATION (DISTRIBUTION %) OF ALBUMIN AND GLOBULINS IN NORMAL

EQUINE SERUM. (Mean \pm standard deviation.)

Group	No.	Globulin fractions (%)													
		Albumin		Alpha-1a		Alpha-1b		Alpha-2a		Alpha-2b		Beta-1		Beta-2	
		\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s
H	25	33.4	4.52	3.3	0.93	4.1	1.00	3.6	0.92	8.5	1.40	17.8	3.42	11.6	2.91
J	10	39.1	3.41	4.4	0.71	4.4	0.71	3.4	0.79	7.4	1.31	16.9	2.62	8.8	2.02
														17.4	3.22
														15.9	1.81

Group H = 2-6 y.o. Eriskay ponies.

J = 2-4 y.o. Thoroughbred and Thoroughbred cross horses.

TABLE 15.4

AGAROSE GEL ELECTROPHORETIC SEPARATION (ABSOLUTE CONCENTRATIONS (g/l)) OF ALBUMIN AND

GLOBULINS IN NORMAL BOVINE SERUM. (Mean \pm standard deviation.)

Group	No.	(g/l)		(g/l)		Globulin fractions (g/l)											
		T. Protein		Albumin		Alpha-1		Alpha-2		Inter Alpha/Beta		Beta-1		Beta-2		Gamma	
		\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s'
A	10	54.8	6.97	20.5	1.83	4.3	1.38	7.7	1.17	2.3	0.49	9.3	1.50	5.0	1.68	5.5	3.04
B	8	61.6	3.11	28.8	2.06	2.0	0.45	5.1	0.57	2.4	0.31	7.3	0.70	7.0	1.34	8.9	1.36
C	8	68.2	4.17	32.9	2.31	1.4	0.54	5.5	0.32	1.9	0.29	6.2	0.37	7.1	0.71	13.8	2.21
D	8	67.6	2.65	31.0	1.67	1.4	0.37	5.1	0.74	2.3	0.36	6.8	1.24	7.5	1.12	14.4	1.93
E	8	67.8	2.77	34.0	2.18	1.2	0.34	5.1	0.39	1.9	0.47	5.6	0.63	7.4	1.20	12.5	1.90
F	8	65.3	4.34	33.2	2.33	2.0	0.54	5.5	1.08	2.3	0.34	6.4	0.80	5.6	1.01	11.3	3.51

Group A = 3-4 w.o. Ayrshire calves.

B = 2 y.o. Blue-grey heifers.

C = 5 y.o. Blue-grey cows, in calf.

D = 10 y.o. Blue-grey cows, in calf.

E = 10 y.o. Blue-grey cows, calved 2 months.

F = 4 y.o. Friesian cows, calved 3 months.

TABLE 15.5

AGAROSE GEL ELECTROPHORETIC SEPARATION (ABSOLUTE CONCENTRATIONS (g/l) OF ALBUMIN AND GLOBULINS IN NORMAL OVINE SERUM. (Mean \pm standard deviation.)

Group		No.	(g/l) T. Protein		(g/l) Albumin		Globulin fractions (g/l)								
							Alpha-1		Alpha-2		Beta		Gamma-1		Gamma-2
			\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}
G	20	81.4	4.02	30.8	2.56	5.1	1.49	9.2	0.91	6.1	1.54	23.8	4.46	5.8	2.03

Group G = 1½-2 y.o. Greyface gimmers.

TABLE 15.6

AGAROSE GEL ELECTROPHORETIC SEPARATION (ABSOLUTE CONCENTRATIONS (g/l)) OF ALBUMIN AND GLOBULINS IN NORMAL EQUINE SERUM. (Mean \pm standard deviation.)

Group	No.	Globulin fractions (g/l)																	
		(g/l) T. Protein		(g/l) Albumin		Globulin fractions (g/l)													
		\bar{x}	s	\bar{x}	s	Alpha-1a		Alpha-1b		Alpha-2a		Alpha-2b		Beta-1		Beta-2		Gamma	
		\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s	\bar{x}	s
H	25	65.6	5.31	21.9	2.34	2.1	0.52	2.7	0.70	2.3	0.62	5.6	1.00	11.7	2.83	7.6	2.03	11.4	2.20
J	10	67.7	4.12	26.3	1.19	2.7	0.36	3.0	0.45	2.3	0.46	5.0	1.15	11.3	2.18	6.0	1.59	10.7	1.04

Group H = 2-6 y.o. Eriskay ponies.

J = 2-4 y.o. Thoroughbred and Thoroughbred cross horses.

The mean and standard deviation of serum protein electrophoretic percentage values in three species were calculated and these are presented in Tables 15.1, 15.2 and 15.3. The mean and standard deviation of total serum proteins and absolute electrophoretic values are presented in Tables 15.4, 15.5 and 15.6.

Discussion

It is clear from Tables 15.1 and 15.4 that the percentage and absolute values for adult cattle in Groups C to F are similar and that variations induced by pregnancy are confined to minor alterations in the albumin and gamma globulin values. In contrast, the percentage and absolute values for the younger animals in groups A and B differ from those in adult animals. Calves have significantly lower total protein levels than adults and the difference is due to lower albumin and gamma globulins, offset to some extent by higher alpha-1 and alpha-2 globulin levels. The figures of James and Polan (1978) for Holstein calves, although differing in absolute terms, support my observations. Their figures for Holstein calves are lower than those given by other authors for adult cattle. (See Table 4.2b, page 47).

Tumbleson et al (1973) recorded breed related differences in total protein, albumin and gamma globulin levels and also age related differences in total protein and gamma globulin levels. My figures from Table 15.4 also show certain age related differences, in that total protein, albumin and gamma globulin levels were all lower in 2 y.o. heifers than in older cows. At first sight there does appear to be a breed related difference (Table 15.1) in albumin and gamma globulin percentage values between Group C and Group F

since the ages are similar. However, these differences can probably be mostly attributed to the fact that Group C is composed of pregnant cows whereas in Group F all the cows are 3 months calved. As stated previously, Rowlands (1978) recorded a positive difference in albumin levels between pregnant and post-parturient cows which appeared to be maximal at 3 months after calving. The earlier figures of Little (1974) are also in agreement with these findings. A similarly timed but negative difference in mean total protein level was also recorded by the former author and although there is some variation between our absolute values, this agrees with my findings in Table 15.4.

From Tables 15.2 and 15.5 it is evident that while the percentage and absolute values of albumin, alpha and beta globulins in adult sheep are comparable with those in the other species studied, the total protein and gamma globulin values were considerably higher. This observation is supported by the figures of other authors in Table 4.3b, page 49 . If my percentage values for ewes from Table 15.2 are compared with those of Perk and Lobl (1960) in Table 4.3a, page 48 , apart from some variation in the numbering of the alpha globulins, the remainder of our values are in good agreement. My figures and those of Ek (1970) in the same table are in good agreement for the alpha-1, alpha-2 and beta globulin fractions, but differ considerably between albumin and gamma globulin values. Unlike myself, Perk and Lobl (1960) and Osbaldiston (1972), Ek did not quote figures for gamma-2 globulin in ewes, although he did recognise that sub-division was possible. Apart from the differences in support media used, some of the differences in absolute values

between my figures and those of other authors could be attributed to variations in age and state of pregnancy. For example, Mackie (1977) found that total protein and albumin levels in intensively bred ewes were affected by age and state of pregnancy, in that during the third pregnancy cycle (commencing at approximately 2 y.o.) the maximum concentrations were reached at 8 weeks gestation. The maximum concentrations in progressive cycles became progressively lower, were reached sooner and in the case of total protein, the decrease in concentration within a cycle became greater as the number of pregnancies increased. Gamma globulins were not affected by the number of pregnancies but were significantly decreased within the cycle. One cycle comprised 144 days gestation, 28 days lactation and 33 days weaning.

If my figures for adult sheep are compared with those for lambs in Tables 4.3a and 4.3b, it is evident that the albumin percentage values for adults are considerably higher and the gamma globulin percentage values are lower than in lambs. In absolute terms, however, the lower total protein levels found in the younger animals have the effect of reducing the albumin difference and accentuating the gamma globulin difference. The figures of the other authors in these tables also appear to be in agreement with these observations. From Table 15.3 it is apparent that apart from a difference in the mean albumin percentage value and to a lesser extent in the mean beta-2 globulin value, the mean values for the other globulins between horses and ponies were comparable. A similar relationship was also noted in absolute terms in Table 15.6, in which the mean total protein value for Eriskay ponies was found to be only 2 g/l

lower than for horses. A breed related relationship between these parameters was also noted by Kelly and Nutter (unpublished data), and in younger horses by Ferraro, Voss et al. (1979), who compared Arabian and non-Arabian total protein and electrophoretic values. As recorded in the other two species discussed, total protein levels in very young animals have been shown to be considerably lower than in adults, although Sato, Oda and Kubo (1979) noted that after the fifth day of life these remained constant, as did albumin levels, over the six months of their study. None of the ages of the subjects in either of my equine groups were less than 2 y.o. or older than 6 y.o. and in the case of group J, all were female. Group H also included a large proportion of female subjects. Salutini and Biagi (1978) found that in trotting mares, the albumin, alpha-1 and alpha-2 globulin levels all decreased with age, whereas gamma globulin increased sharply from 1-6 y.o. and then gradually decreased.

Although none of the subjects in Groups H and J were pregnant, certain changes in the serum protein levels of pregnant mares have been recorded and are worth mentioning at this point. Jaeschke and Muller (1975) noted that total protein values exceeded the normal limits only in the first 5 days after parturition. The concentration of alpha-2 globulins decreased during the last third of pregnancy and increased during the first 5 days of parturition to reach a maximum at 30-45 days. Beta and gamma globulin levels on the other hand did not change significantly.

If my results for horses in Table 15.3 and 15.6 are compared with those of Kristensen and Firth (1977) in Tables 4.4a and 4.4b, apart

from some disagreement in the numbering of the alpha globulins, which makes direct comparison less straightforward, the remainder of our figures were in good agreement. Although using the same support media as the latter authors and myself, Liberg et al. (1977) (Table 4.4b) performed electrophoretic analysis on their own water cooled plates, with greater gel thickness than of the commercially prepared agarose films. They found that since the albumin zone became relatively heavily stained after processing, the albumin concentration was usually underestimated by their gel scanner. Consequently they ignored all albumin values and reported the relative values of globulins only.

The percentage values for horses quoted by Pierce (1975) in Table 4.4a are in good agreement with my figures for horses in Table 15.3. Pierce was not able to differentiate alpha-1, alpha-2 or beta globulins, but if my sub-fractions for each of these zones are merged, our corresponding values are very close. In the same table, the albumin and gamma globulin values of Kao et al. (1954) for horses are similar to my values in Table 15.3 but there are wide variations between the remainder of the fractions. Apart from good agreement with the albumin figures of Rose & Paris(1979), comparison between my figures and those of other authors in Table 4.4a are less favourable.

Other authors in Table 4.4b quote total mean protein values which range from 61.0-75.6 g/l, albumin values from 23.9-32.9 g/l, beta-1 globulin values from 6.3-19.6 g/l and gamma globulin values from 8.2-15.0 g/l, thus demonstrating a wide variation in 'normal' values.

It is clear that in some cases there are wide differences in each species between some of the normal electrophoretic values which have been reported. For reasons which have already been outlined, the choice of support media, breed, age and stage of lactation are only a few of the factors which are likely to have an influence on the levels of various protein fractions after electrophoresis. It must therefore be emphasised that any comparison between any set of electrophoretic protein values should be undertaken with caution. If possible, normal values should be determined for the particular electrophoretic method to be used in the laboratory, with the relevant details of each group of animals clearly specified.

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